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Molecular Detection of ~~Sclerotinia~~ Ascochyta Resistance Genes for Lentil Breeding

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SASKATCHEWAN AGRICULTURE DEVELOPMENT FUND

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MOLECULAR DETECTION OF ASCOCHYTA RESISTANCE GENES FOR LENTIL BREEDING

FINAL REPORT April 1992

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ABSTRACT

DNA from lentil (*Lens culinaris* L.) genotypes was examined for variability in both Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) markers, in a search for molecular markers which might be genetically linked to one or more of the genes responsible for the high level of resistance to *Ascochyta* blight found in the Syrian accession ILL5588. From 536 possible RFLP tests, only four potential markers were found, but a possible 17 RAPD markers were found from 140 oligonucleotide primers tested. Individual parent and F_2 plants from a cross between the susceptible Saskatchewan-adapted cultivar Eston and a Syrian accession of lentil (ILL5588) which carries two genes for resistance to the pathogen, were grown in the Crop Development Centre's disease nursery and inoculated with crop residues containing *Ascochyta*. Shoot tissues were collected during the season and DNA was prepared and tested with these markers. As well, the level of *Ascochyta* infection was determined by checking the seed from individual plants at the end of the season. In the F_2 population all but one of the markers had the expected segregation ratio and the distribution of *Ascochyta* infection was compatible with the current understanding of the genetic system controlling the resistant phenotype. Although the gel bands produced by RAPD markers do not qualitatively discriminate between homozygous and hemizygous genotypes, as do RFLP markers, some preliminary results suggest that quantitative densitometry may make it possible to distinguish them. Comparison of the disease scores in the F_2 plants with the RFLP and RAPD markers identified one RFLP and three RAPD markers which are good candidates for use in selecting for the *Ascochyta* resistance genes. These markers should be tested with larger segregating populations or advanced breeding lines.

TECHNICAL REPORT

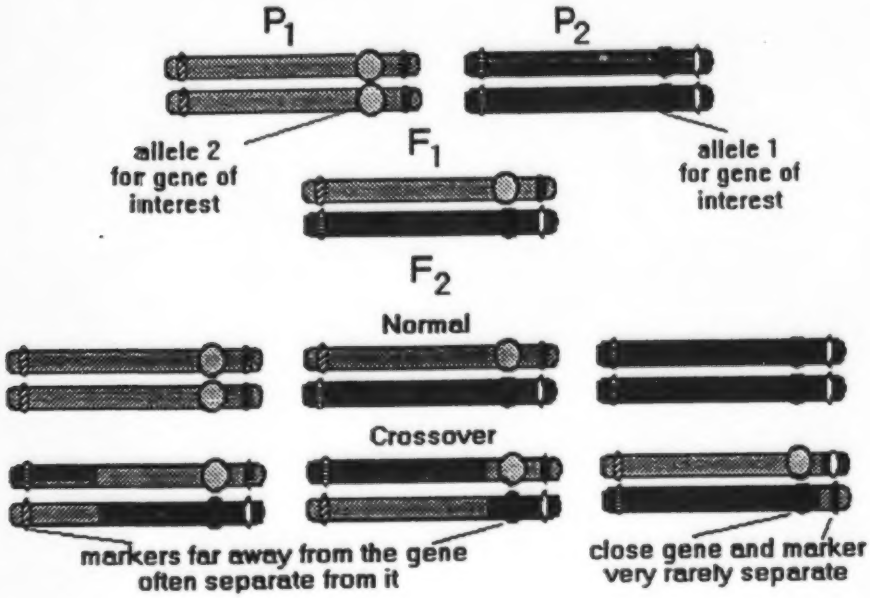
1. Nature and Scope of the Problem

Ascochyta blight causes major economic losses for producers of lentil (*Lens culinaris* L.) in western Canada. It can appear at any stage of development and can contribute to leaf loss, stem weakening and necrosis of the shoot tips, any of which can decrease yield. Infection of the pods and seeds can also affect yield, but of equal importance, it can decrease the seed quality and thus the economic return (Martens et al. 1985). Although the Saskatchewan-bred variety, Laird, has some genetic resistance to the disease, it does not persist throughout the growth cycle. Recently, another source of genetic resistance to the disease has been found in a Syrian accession (Erskine et al., 1988). Lines ILL5588 and ILL5684, derived from this accession, have been crossed with Laird and Eston lentils both to study the genetic control of the resistance and to initiate a program of introgressing the resistance into cultivars adapted to western Canada (Tay, 1989). This process is always time consuming, but when fungal resistance is involved, it can be especially difficult, as the development of disease symptoms is affected not only by the plant genotype, but also by the available inoculant and the environmental conditions throughout the season. The setting up and operation of a disease nursery for assessing the response of breeding material to fungal pathogens requires time, space and money, and even in nurseries, climatic conditions can be such that disease symptoms cannot be detected. Under these conditions, the season may be lost with respect to evaluation of the material in that selection cycle.

For many years, geneticists and plant breeders have used genetically linked markers to follow the segregation of traits which are difficult or expensive to evaluate. This works on the general principle that traits determined by genes located on the same chromosome tend to be inherited together. When parents differ in both the gene of interest and a more easily identified gene (marker gene) on the same chromosome, the expression of the marker will indicate the presence of the allele of the important gene with which it is associated in a parent. Although, as illustrated in Figure 1, the occurrence of crossover events during meiosis can separate genes on the same chromosome, the closer two genes are on a chromosome, the higher the probability that they co-segregate, so that for all practical purposes, a closely linked marker allele indicates the genotype. Nevertheless, the expression of markers such as single-gene morphological traits (colour, shape) or biochemical traits (chemical resistance, protein patterns) is somewhat removed from the actual presence of the gene. Environment can affect the detection of even these genes, some may not be obvious until late in the season and dominance problems can make it difficult to distinguish between homozygous and heterozygous plants.

Recent developments in molecular biology have produced new genetic markers with which specific chromosomal locations can be detected directly on the basis of the DNA sequences. The first such marker system was the Restriction Fragment Length Polymorphism (RFLP) which is now being used for many purposes. Since then, several other molecular methods have been developed. These methods have received widespread publicity through their potential to generate "genetic fingerprints" for forensic purposes and because of their successful use in "chromosome" walking from known marker genes to find the genes responsible for human genetic disorders. There are , however, a number of possible problems which might limit the utility of such "molecular markers" in the area of plant breeding where resources are more limiting than in these more medically oriented examples.

Figure 1. Diagrammatic representation of how a specific gene of interest and marker genes, located at different distances from it on the same chromosome, behave during two generations of normal controlled pollination.



2. Objectives of the Project

This research project was developed with the general objective to **develop a procedure for the detection of disease resistant plants, even under disease-free conditions using restriction fragment length polymorphisms (RFLP's).** The specific technical objectives were:

a) to develop DNA probes which can detect genetically variable restriction fragments in lentil genotypes.

b) to identify combinations of probes and restriction digests producing fragment patterns with genetic linkage to the *Ascochyta* resistance trait.

c) to develop the complementary methodology for routine screening of at least 50 plants per week.

The research plan was submitted to the Saskatchewan Agricultural Development Fund in June 1989, it was approved in principle and a contract for the research (R-89-05-0480) was signed in January, 1990.

3. Research Approach

For this work, several lentil genotypes were collected, including varieties which had already been used for RFLP mapping (Havey and Muehlbauer, 1989), two varieties adapted to Saskatchewan growing conditions, two accessions from Syria with resistance to *Ascochyta* and some selected lines of known response to *Ascochyta* (Tay, 1989).

The research plan aimed at using RFLP technology to find polymorphic markers in lentil genotypes, and the theoretical basis of this type of marker was discussed in some detail in Progress Report #1.

DNA from the collected genotypes was to be extracted, enzymatically cleaved with several restriction endonucleases, separated according to size by agarose electrophoresis and transferred ("blotted") onto membranes. The DNA on these membranes would be hybridized to many radioactive DNA probes obtained from a cDNA library produced by a commercial company from RNA extracted at PBI from Laird lentil plants. Probe-enzyme combinations which revealed fragments of different lengths corresponding to the known response to *Ascochyta* blight, were to be used to examine the F_2 progeny plants of a resistant x succeptible lentil cross.

Throughout the course of this work, methodology was to be developed with a view to decreasing the cost, the labour, the hazard and the amount of plant tissue required so as to facilitate the transfer of this technology to practical breeding programs.

A Research Scientist and a Technical Assistant were hired to carry out the experimental and data handling tasks, under the supervision of Dr. John Mahon. Drs. A. Slinkard and S. Hobbs collaborated and the project was scheduled for two years.

4. Modifications to Original Plan

Several modifications were made to the work plan during the course of the contract, in response to unexpected results, new methodologies and organizational changes. Of these, three had major impacts on the research approach and the work schedule.

a) Personnel Changes

In April 1991, the Research Scientist in charge of the work, Dr. Om Rajora, left PBI to take up a new position in Edmonton. This was at a critical time in the work when examination of the new cDNA library was beginning. Because of the difficulties in obtaining the services of a Postdoctoral Scientist with experience in this area for a one year term appointment and the scheduling constraints of the work, the decision was made to replace Dr. Rajora immediately with an M.Sc. graduate having experience in RFLP methodology, Mr. Jiyue Liu. We were very fortunate to obtain the services of a such a well qualified scientist who arrived in June, 1991. Although Mr. Liu had to catch up on progress in the project and learn new methods, he and Mr. Kindrachuk were able to maintain a good rate of progress. Nevertheless, additional time for completion was requested and granted. A second consequence of this staffing change was that a much greater proportion of the scientific planning and evaluation had to be taken on by Dr. Mahon.

b) Introduction of New Technology

In the fall of 1991, difficulties were being encountered with a low level of genetic variability being detected in the lentil genotypes with the probes isolated from the new

cDNA library. At the same time, discussions with other scientists involved in lentil work, suggested that genetic polymorphisms might be more easily found with a new method based on Random Amplified Polymorphic DNA markers (RAPD's) (Williams et al., 1990). Therefore, in collaboration with Dr. L. Pelcher of the DNA Services Unit of PBI, this approach was tried in parallel with the RFLP methods.

c) Lack of Disease Development in the Field

As discussed in the introduction, fungal disease symptoms may not develop if inoculation or climatic conditions are not suitable. As reported in Report #3, during the summer of 1991, overall symptom development was poor and disease ratings in the field could not even distinguish between the resistant and susceptible parents. After consultation with pathologists Drs. Morrall and Peterson (Department of Biology, University of Saskatchewan, Saskatoon), the seeds collected from individual parent and F_2 plants, from which tissue samples had been harvested for DNA preparation, were screened indoors for *Ascochyta* (Morrall and Beauchamp, 1988). This procedure required a great deal of Mr. Kondrachuk's time when the RFLP screening was getting underway. However, as very good results were obtained for both parents and F_2 plants, the much more difficult and time consuming inoculation and symptom assessment in controlled environment cabinets was avoided.

5. Methods and Results

a) Plant Material

The major genotypes of lentil (*Lens culinaris* L.) used for this project were cv. Brewer, cv. Redchief, *Lens orientalis* sp. (from Dr. F. Muehlbauer, USDA/ARS Grain Legume Program, Pullman, Washington), cv. Laird, cv. Eston, accessions ILL5588, ILL5684 and F_5 seed of lines from Eston x ILL5588, ILL5588 x Eston and Laird x ILL5588 crosses (from Dr. A.E. Slinkard, Crop Development Center, University of Saskatchewan, Saskatoon, Sask.). These F_5 lines had been evaluated individually for both foliar and seed *Ascochyta* symptoms in both the F_3 and F_4 generations (Tay, 1989). Seeds from three individual plants of each of Eston, Laird, ILL5588 and ILL5684 were obtained, grown separately and compared in order to detect any genetic heterogeneity.

Plants were grown for seed multiplication and controlled crossing in a controlled environment chamber (Enconaire, Winnipeg, Man) at day/night temperatures of 20/15 C and a 16 h photoperiod at 500 μmol quanta m^{-2} s^{-1}, provided by equal numbers of 400 W Metalarc and Lumolox HID lamps. Five seeds were planted into 15 cm plastic pots of Turface mixed with 15 g of Osmocote slow release fertilizer (14-14-14). Plants were irrigated with tap water daily and with nutrient solution every 2 weeks.

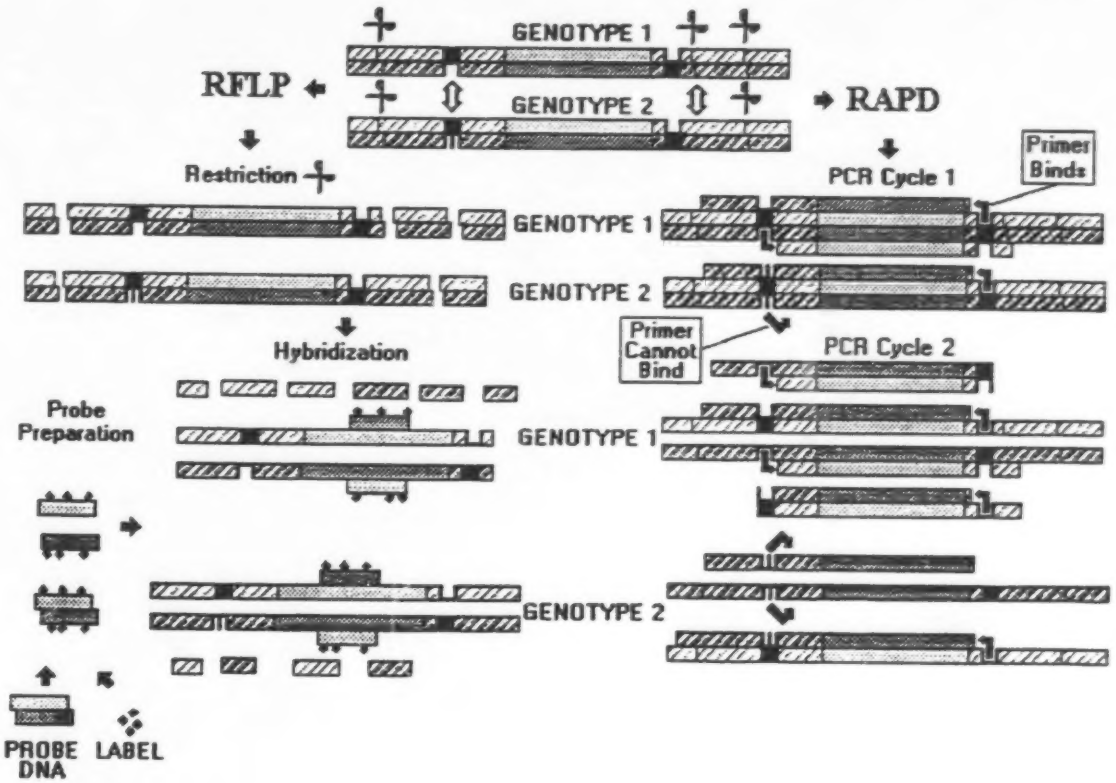
For controlled crosses, plants of all parents were planted in three groups at 2 week intervals to ensure the presence of flowers on both parents needed for each cross. Flowers of the female parent were opened, examined for anther dehiscence and if no free pollen could be detected, emasculated. Pollen from dehiscing anthers of flowers from the male parent was dusted onto the stigma of the emasculated female parent and the flower was labeled. In most cases, reciprocal crosses were carried out and mature pods were harvested individually by hand, as progeny from different crosses could be on the same plant. For producing F_2 progeny, individual F_1 seeds were planted separately and each F_2 population, grown in the field for assessing *Ascochyta* infection, was from a single F_1 plant. All F_1 plants from crosses between ILL5588 and either Eston or Laird were analyzed by RFLP technique using a specific probe which produced different bands for the 3

genotypes. The presence of the correct two bands in Southerns demonstrated that the plants were indeed F_1 plants rather than the result of self pollination of the maternal parent. A figure showing these results was presented in Report #3.

b) Molecular Gene Markers

As discussed above, the project eventually compared two different methods of detecting polymorphisms based on differences in DNA sequence. Much of the theory behind the generation of RFLP markers was discussed in Report #1, and the RAPD markers are another spin-off from the same Polymerase Chain Reaction (PCR) technology which was used to produce λgt11 inserts in high amounts as described in Report #3. Nevertheless, Figure 2. provides a diagrammatic comparison of how the RFLP and RAPD methods produce detectable genetic polymorphisms from simple differences in DNA sequence.

Figure 2. Diagrammatic representation of the molecular basis for producing visible polymorphisms from the DNA of different genotypes using RFLP and RAPD technologies.



The chromosomes of genotypes 1 and 2 at the top of the figure are the same in sequence except for two simple (perhaps single base pair) differences shown by the open two headed arrows. One of these differences eliminates a cleavage site in genotype 2 for the restriction endonuclease represented by the scissors and produces an RFLP. The other sequence change prevents the binding of a short oligoncleotide (9 or 10 bases) primer to one of the sites and is the basis of RAPD's.

The left side of Figure 2 shows the steps in RFLP technology. Restriction digestion of the DNA produces one less fragment and one longer fragment in genotype 2. These fragments can be separated according to size by electrophoresis and the DNA transferred to a membrane. Probe DNA with a sequence similar to that of a part of this chromosome can be radio-labeled and will hybridize to the complimentary sequence on the long fragment from each genotype to show bands at different positions on the gel.

On the right side of Figure 2 is the basis of the RAPD method. If a primer can bind to 2 sites on opposite strands of chromosomal DNA within a sufficiently short distance from each other for the PCR reaction to work, as in genotype 1, then in the first synthesis cycle, a copy of each strand will be made. In genotype 2, however, a sequence difference prevents the binding of the primer to one of the strands, so that during synthesis only one strand is copied. In the second cycle, genotype 1 now has 2 copies of each strand, all with good primer binding sites. This leads to a doubling of the number of copies and, as well, to the beginning of an enrichment in fragments of a length corresponding to the length including the 2 primer binding sites. For genotype 2, however, of the 3 strands in the reaction mixture after the first cycle, only one has a usable binding site, so that only one strand is copied. This produces an additive increase in DNA as compared to the exponential increase from genotype 1 DNA and no production of fragments of a length corresponding to the distance between the primer binding sites.

c) *Ascochyta* Assessment

The utility of a genetic marker is directly dependent on the extent to which it is genetically linked to the trait of interest, in this case, the resistance to *Ascochyta* blight. The closeness of this linkage can only be determined by comparison of the expression of the markers with the sensitivity to the disease in different members of a segregating population (usually F_2 population, although doubled haploid lines from F_1 plants are preferable when they can be produced). To generate such material, crosses were made in a controlled environment chamber between Eston or Laird and ILL5588 (both reciprocals). The progeny were tested for RFLP evidence that they were indeed hybrid plants and the F_2 seed, as well as seed from all parental lines, were planted in the disease nursery operated by Dr. A.E. Slinkard of the Crop Development Centre, University of Saskatchewan. The six lines were planted as described in Report #2 in 2 replicate blocks containing the 6 single rows. Although shoot material was gathered from plants from an average of over 60% of the seeds planted (during 3 collections), assessments of visual symptoms of *Ascochyta* disease development at two times during the season could not distinguish between the highly resistant parent ILL5588, and the susceptible cultivars Eston and Laird. Nevertheless, seed was collected from as many plants as possible as they matured.

After consultation with pathologists, Dr. R. Morrall and Dr. Eric Peterson of the University of Saskatchewan Biology Department, it was decided to test the seed for the presence of seedborne *Ascochyta* which might indicate infection late in the season. Seed lots were selected on the basis of large numbers of seed in which to test for the presence of *Ascochyta* (>50 seeds per plant) and the amount of shoot tissue that had been collected for DNA extraction during the season (at least 2 x 1 g samples). The procedure used was a modified form of that used by Morrall and Beauchamp (1988). The seeds were surface sterilized and then plated 10 per petrie plate on V8 + streptomycin agar. After 10 to 14 days, the dishes were examined under a dissecting binocular microscope for *Ascochyta* growth (with its characteristic colour and pycnidia). As many plates were done for each plant as there were seeds available, up to a maximum of 12 plates, but no more than 4 plates were prepared from a plant for a single experiment. Data were collected for all 6 genotypes; however, because of poor survival of the Laird and the F_2 progeny from Laird x

ILL5588, only the data from Eston, ILL5588 and the combined plants from both reciprocal crosses of Eston x ILL5588 are presented in detail in Appendix 1 and summarized in Table 1. The individual plants within Eston and ILL5588 did not differ significantly, but the

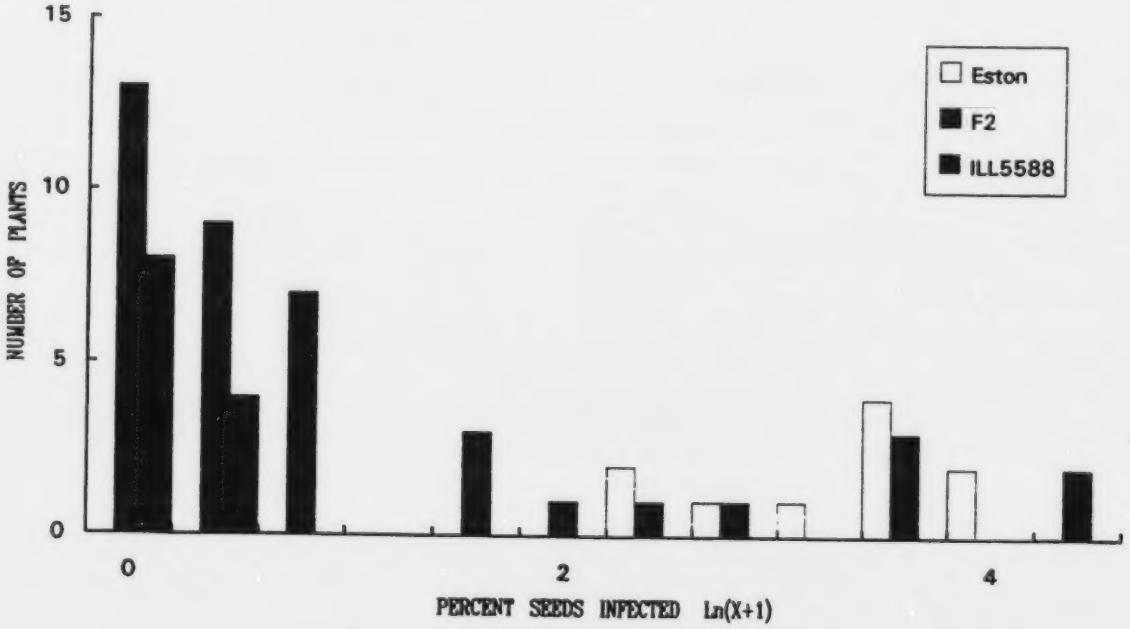
Table 1. Summary of *Ascochyta* infection assays on seed collected from parental and F_2 progeny generations of lentil.

POPULATION	PLANT NUMBER	MEAN PLATE NUMBER	PERCENT INFECTION[1]	BETWEEN PLANT VARIABILITY[2]
ESTON	10	10	41.2 a	n.s.
ILL5588	12	8	3.2 c	n.s.
ESTON x ILL5588	40	11	14.6 b	P<0.001

[1] Data transformed as arcsin $\sqrt{}$proportion infected, different letters show significant differences (P<0.05).
[2] n.s. - individual plants did not differ significantly (P<0.05)

means for the 2 genotypes did. However, individual plants within the F_2 population were significantly different, ranging from 0 to 71%. Disease response is often rated on a logarithmic scale and the distributions of the individual plant values, expressed in this way (Figure 3), show the broad range of values for the F_2 plants. This apparent segregation was supported by the significantly greater "between plant" variance in the F_2 population,

Figure 3. Distribution of parent and F_2 plants on the basis of *Ascochyta* presence in seeds harvested at the end of the 1991 field season.



as compared to either of the parent groups. The genetic model proposed for the genotypes used here (Tay, 1989) suggests that ILL5588 differs from Eston in that it carries 2 dominant genes (Ral2, Ral3) for both foliar and seed resistance to *Ascochyta*, as well as a recessive gene (ral1) that confers some foliar resistance. The resistance associated with this latter gene, however, is said to break down in the late podding stages and is thus unlikely to influence the assessment of disease severity in this study, which is based solely on seed infection. While the number of plants assessed in this study is too small for any rigorous genetic interpretation, it is worth pointing out that the distribution of the F_2 plants is not incompatible with simple genetic models. Table 2 shows theoretical and observed distributions for one and two gene models with additive gene action. In the single gene

Table 2. Theoretical and observed distributions of 40 F_2 plants from a cross between Eston and ILL5588 lentils. Expected numbers are based on one or two independent additive resistance genes and observed values are grouped into arbitrary classes on the basis of seed infection.

GENE NUMBER	NUMBER OF F_2 PLANTS	NUMBER OF GENE COPIES PRESENT				
		4	3	2	1	0
1	EXPECTED			10	20	10
	OBSERVED			11	20	9
	CLASS RANGE			0	0 - 1	> 1
2	EXPECTED	2.5	(12.5)	10.0	15	10	2.5
	OBSERVED	--------	13	--------	16	9	2
	CLASS RANGE	-----	0 - 0.2	-----	0.2-1.6	1.4-4.0	> 4.0

model, it was easy to define classes of seed infection, using ln transformed data, which fit the model well (Table 2). However, in the case of the two-gene model, there were 9 F_2 plants with no *Ascochyta* infection, and this could not fit the genetic model unless the 3 and 4 copy genotypes were assumed to confer similarly high resistance, both equivalent to that in ILL5588 (7 plants = 0, 5 plants <0.6).

Although this F_2 population may be too small for genetic analysis or for resolving genetic linkage with high precision, the 40 plants exhibited a wide range of response to *Ascochyta*, good reproducibility between independent plates of seed tested from each plant and a distribution that was compatible with simple genetic models. It was considered that these F_2 plants would be suitable for obtaining the necessary information on the relationships between the molecular markers and the genes controlling the resistance trait.

c) Extraction of Plant DNA

The basic material needed for both RFLP and RAPD molecular markers is DNA from the plants whose genomes are being studied. However, while both of these methods require DNA, the enzymatic reactions used to produce the visible polymorphisms are different and the quality of DNA needed to produce good results may differ. During the course of the project, considerable effort was made to develop appropriate methodologies. Results from testing different methods of extracting large amounts of DNA from leaves of lentil plants grown in controlled environment conditions, are mentioned in Reports #1 and #2 and only the final method used for preparations of 0.5 to 1.5 g fresh weight of leaf tissue (between 0.2 and 1 mg DNA) is presented in Appendix 2. This method was used for extracting DNA for the initial screening of resistant and susceptible genotypes from all of the lentil genotypes grown indoors. The method was also used to extract DNA for RFLP

and RAPD analysis of individual plants from parent and F_2 generations of an Eston (susceptible) x ILL5588 (resistant) cross. From these plants growing in the field, shoot material was harvested from individual plants (≈ 1 g fresh weight) at 3 times during the season. Leaflets and shoot tips were collected into plastic vials and were immediately quick frozen on dry ice for transport to the laboratory where they were stored at -70C until DNA was extracted from 0.5 g fresh weight samples. DNA was extracted from a total of 4 plants of each parent (Eston, ILL5588) and the 40 F_2 plants.

When polymorphic markers are to be used to screen individual plants for the presence of a molecular marker that has already been shown to be linked to the trait of interest, different methodology is needed. In this case, less plant material may be available (if very young seedlings are to be used), less DNA will be required because fewer enzyme/probe combinations (RFLP) or primers (RAPD) need to be examined, samples may have to be collected in the field and perhaps stored for long periods of time before extraction or analysis and very large numbers of samples may have to be handled and stored. Because the development of this methodology was one of the project goals, leaflet samples of 2 or 4 leaflets from each of two lentil genotypes (mean fresh weights = 15 ± 3 (Laird) and 12 ± 3 (Eston) mg fresh weight per leaflet) were extracted by modified versions of the following three methods: Phenol/Chloroform, Dellaporta, CTAB (Appendix 2). The Phenol/Chloroform and CTAB methods were tested with and without addition of polyvinylpyrrolidone. Samples of the final products from all methods were run on a 1% agarose gel in TAE buffer along with 1.6, 0.4, and 0.1 μg of CsCl-purified plant DNA as standards. The ethidium bromide - stained gel was photographed with a Polaroid system using Type 55 film to produce a negative which was scanned with a Molecular Dynamics Scanning Densitometer. DNA contents of the samples were estimated by comparison of the integrated values for samples with those of the standards. There were no significant effects of the sample size (number of leaflets) or the addition of polyvinylpyrrolidone on either the recovery of DNA or the amount of ethidium bromide - staining material which

Table 3. Effect of extraction method and genotype on recovery of DNA and percent of ethidium bromide - staining material remaining in loading well.

Factor	DNA Recovered ng mg^{-1}	DNA Remaining in well %
Method		
Phenol/Chloroform	185	10
CTAB	237	28
Dellaporta	248	28
LSD .05	ns	4
Genotype		
Laird	249	22
Eston	188	20
LSD.05	41	ns

did not migrate through the gel (Table 3). More DNA was recovered per unit of leaf weight from Laird than from Eston lentils, although it seems likely that this is related more to the morphological differences than the actual content of DNA. The amount of ethidium bromide - staining material remaining in the wells of the gel was less in DNA from the Phenol/Chloroform method and was similar to that seen in the CsCl purified plant DNA used

± 4%). In general, extraction of 4 lentil leaflets by any of these methods enough DNA for 2 Southern blots or 50 PCR amplifications. More than 50 mples of this size could be collected in one day by a single worker in the field, but in order to facilitate documentation and storage of samples, two people would be preferable. Depending on facilities available, DNA could also be routinely extracted from 30 samples by one person in a single day. Nevertheless, it must be emphasized that during the development phase of the work, when DNA must be tested with many potential markers, larger samples are necessary.

Although all of these mini methods yielded satisfactory quantities of DNA, Figure 4a ows that the most reproducible and best quality PCR products were obtained from the A prepared by the CTAB method (without and with PVP). DNA prepared by the ified Dellaporta method was intermediate in its ability to produce the diagnostic bands both genotypes) and DNA from the Phenol/Chloroform method was rarely successful. or RAPD markers, the CTAB method appears to be the best method. Analysis of the o cut these DNA samples with a restriction enzyme (Figure 4b) indicates that the duced by all methods was equally good (as shown by the similarly wide n in fragment sizes).

n of RFLP Markers

riginal work plan, RFLP were the designated molecular markers being sought in mapping some RFLP markers success of Havey and Muehlbauer (1989) in Reports #1 and #2 with the probes provided over, the results presented in Reports #1 and #2 with the probes provided r, indicated that some of his probe/enzyme combinations produced RFLP's d between pure lines known to differ in resistance to *Ascochyta* blight.

in Reports #1 and #2, different procedures for DNA restriction horesis of DNA samples, transferring DNA from agarose gels to and hybridizing radioactive probes to the DNA immobilized on 1. Only the single set of protocols producing the optimal RFLP s the methodology of choice in Appendix 3. The cDNA library was

ns produced from DNA extracted from small samples of til leaf tissue by 5 methods. a. PCR products produced r (U563) and DNA samples. The four lanes in each aflets; Laird, 4 leaflets; Eston, 2 leaflets; Eston, 4 b. agarose gel of restriction (BglII) digests of DNA s. Within each group are combined samples of 2 and Laird and Eston respectively.

produced by Clontech Laboratories Inc. from RNA extracted at PBI. The library, containing cDNA fragments cloned into the EcoRI site of a λgt11 cloning vector was as described in Report #2. The library was plated out and individual plaques were stored for future use. The protocols for isolating cDNA inserts from the λgt11 clones are also presented in Appendix 3.

In order to screen a large number of cDNA clones, many identical blots were prepared. For each, DNA prepared from 7 lentil genotypes of known response to Ascochyta (S=susceptible; R=resistant), Laird(S), Eston(S), ILL5588(R) and ILL5684(R), as well as three evaluated breeding lines L23(S), L29(R), L35(R) was used, Samples digested with BamHI, BclI, BglII and DraI were run on the upper level and with EcoRI, HindIII, KpnI and ScaI on the lower, both with 2 size standards of λDNA cut with HindIII. As RFLP markers are defined by both the probe and the restriction enzyme used, each gel produced 2 blots and a total of 8 possibilities for polymorphisms.

As can be seen in Table 4, the recovery of RFLP markers which differed among the 7 genotypes tested was very low, accounting for only 10 % of those probe/enzyme combinations tested. Moreover, most of these were detected with probes obtained from Dr. Muehlbauer. As these probes had been selected for their ability to show polymorphic patterns in lentil and related species of *Lens*, it is perhaps not surprising that a high proportion would show different patterns in the collection of genotypes used. Very few useful probes were obtained from our screening of the cDNA library. Recovery of useful probe DNA was particularly poor with the PCR method because in 8 of the 10 cases

Table 4. Summary of results with probe/restriction digest combinations tested for suitability as RFLP markers to distinguish between *Ascochyta* resistant and susceptible genotypes. Data are presented as numbers of potential markers and percent of total tested in parentheses.

PROBE SOURCE	COMBINATIONS TESTED	BANDS PRESENT	NOT REPETITIVE	GENOTYPE VARIABILITY
Muehlbauer	104	88 (85)	88 (85)	32 (31)
Clontech library				
λ Insert isolation	128	88 (69)	64 (50)	0 (0)
PCR	80	72 (90)	8 (10)	1 (1)
Plasmid Clone	224	144 (64)	112 (50)	10 (4)
Total	536	392 (73)	272 (50)	43 (8)

tested, hybridization to the Southerns produced bands on the autoradiograms within 10 minutes of exposure, of far greater intensity than could be achieved by hybridization to a single copy nuclear gene. These strong bands were always the same in the 7 genotypes and in some cases, identical patterns were seen in all the restriction digests for different probes. It seems likely that these probes must have hybridized to repeated DNA sequences to have produced such strong signals. It is not known if these signals were related to the additional 163 λgt11 base pairs included in these probes, but as similar strong signals were detected in a few cases with probes produced from EcoRI fragments of λgt11 eluted from gels, there may have been some sequences complementary to highly repetitive DNA regions cloned into the library. By far the best approach was to move the inserts into a plasmid vector and to retrieve the inserts from these. Using this method, only 4% of the probe/enzyme combinations produced polymorphisms; however, reference to Havey and

After size separations on agarose gels, the products of PCR reactions with the single primers appeared as 3 to 40 bands of varying intensities and sizes (300 to 5000 bp). For the purpose of markers, only relatively strong or well separated bands, which were easily identified by eye, were selected. Figure 6 shows a gel with three bands that differed between the parents and segregated in the F2 generation. Of the two kinds of primers tested, the 9- and 10-mers both produced similar proportions of clear band patterns (Table 5). However, the 10-mers produced proportionately more potentially useful polymorphic

Table 4. Assessment of PCR primers for generation of RAPD genetic markers to identify lentil plants susceptible or resistant to *Ascochyta* blight.

PRIMER SOURCE	NUMBER TESTED	CLEAR BANDS	GENETIC VARIABILITY	F2 TESTS	POSSIBLE MARKERS
pont (9-mer)	40	38	21	3	1
(10-mer)	100	88	27 (32)•	14 (19)	10 (15)
	140	124	48 (53)	17 (22)	11 (16)

bers in parentheses indicate total number of polymorphisms as some primers more than one polymorphic band.

ially as more than a single polymorphic band was sometimes produced from a In most cases, the susceptible and resistant genotypes differed by a single l be caused by the inability of a primer to anneal to one of the two sites amplification. Bands of different sizes might be expected in cases where n between the two primer sites of one genotype. However, there was no fragment length differences, because when primers produced more than band, there was never the negative correlation between the presence of

aph of PCR (U516) products from two plants of Eston (4,5) and 7) and 20 F2 progeny. Products were separated on 1.2% ed and photographed during exposure to ultraviolet light.

two bands in the F_2 population as would be expected if the two bands represented different alleles of the same DNA region. A total of 17 primers were tested on parent and F_2 plants from the Eston x ILL5588 cross. In 5 of these, the PCR products were only weakly amplified or produced resolvable bands in too few samples (< 20 F_2 plants). However, even in the best cases, some DNA samples did not produce good bands (Figure 6). Because DNA prepared using different methods did not improve the situation, it seems likely that the problem lay in the original sample collection in the field. Some of the primers produced more than one distinguishing band (Figure 6, arrows), and a total of 17 clearly visible marker bands were examined for segregation patterns. Only one showed a segregation pattern that differed significantly ($\chi^2 = 8.7$, $P<0.01$) from the expected ratio of 3 (band present) : 1 (band absent) which would be expected if amplification of both the single copy in a hemizygous genotype and the 2 copies in a homozygous genotype produced the same band. Rejecting this single marker, left 16 potential markers to be examined for linkage to the *Ascochyta* resistance trait. The names of these markers, the estimated lengths of the diagnostic PCR products, the primers required to produce them and the expression in the original 4 genotypes used to screen for RAPD markers are shown in Table 5.

Table 5. Characteristics of 16 RAPD markers showing different banding patterns between Eston and ILL5588 lentils and no significant deviation in segregation ratios from the expected 3:1 ratio.

PRIMER		RAPD	BAND SIZE	GENOTYPE EXPRESSION			
NAME	SEQUENCE			ESTON	LAIRD	5588	5684
RC41	ACAGCACCC	RC41	680	1	1	0	0
U516	AGCGCCGACG	516	1220	0	0	1	1
		516.2	1490	1	1	0	1
		516.3	1760	1	1	0	1
U519	ACCGGACACT	519.1	1990	1	1	0	0
		519.2	660	1	1	0	0
U521	CCGCCCCACT	521	540	1	1	0	0
U535	CCACCAACAG	535	1200	0	0	1	0
U540	CGGACCGCGT	540.1	500	1	1	0	0
		540.2	1070	0	0	1	1
		540.3	1280	0	0	1	1
U562	CAAAGTAGCC	562	1200	0	0	1	1
U573	CCCTAATCAG	573	320	1	1	0	0
U584	GCGGGCAGGA	584	680	1	1	0	0
U585	CCCGCGAGTC	585	730	1	1	0	1
U599	CAAGAACCGC	599	1130	1	1	0	0

6. Genetic Markers and *Ascochyta* Resistance

Because both the RFLP and the RAPD markers can detect genetic differences of only a single base pair, there is a good chance that polymorphic markers will be found even in relatively closely related plant material. As well, both directly detect differences in DNA sequence, thus eliminating the problems with methods that require high levels of gene expression or that require specific environmental conditions or developmental stages in order to be detected. Indeed, it is precisely because many agronomically valuable genetic traits are so difficult to assess, that linkage markers are sought, so that indirect selection

can be used when the trait itself is poorly expressed or difficult to determine. The difficulty in working with genes controlling a plant's response to fungal pathogens was very clearly illustrated by the inability to detect any disease symptoms during the 1991 growing season, despite the fact that the plants were grown in a disease nursery with inoculation and irrigation.

There are, however, some problems with molecular markers, and the general aim of the work was to see how difficult it is to develop markers in response to the discovery (or acquisition) of plant germplasm with new disease resistance characteristics. The need to work with such new disease resistance genes is fairly common in breeding programs, and the starting materials are likely to be, as was the case in this work, "pure breeding" lines of plants differing in their response to the pathogen. Some information about the genetic system controlling the response will also be necessary.

The first task is to find large numbers of visible traits which differ in a consistent way between those genotypes possessing the desired resistance trait and those into which the controlling genes are to be moved. In our work, an examination of 536 restriction enzyme/probe combinations produced 43 genetically variable banding patterns. However, this is a bit misleading as 75% of these were detected using the probes acquired from Dr. Muehlbauer's group, and these had already been selected from a much larger population of probes for their suitability in detecting polymorphisms in lentil material. It is not known if there was a problem with the cDNA library constructed for this project or if the germplasm was too similar to find many independent markers. The contribution of the RFLP approach was four potential markers showing proper segregation ratios, but two of these (same probe) gave identical segregation results. In our hands, the RAPD approach was more fruitful and 16 potential markers were obtained from 140 primers tested. In two cases, single primers revealed three bands which distinguished between the resistant and susceptible parents. All of these showed appropriate segregation ratios with each marker segregating independently.

In addition to this difference in the frequency of polymorphisms within our lentil genotypes, there are also technical considerations relating to the detection of the two kinds of markers. In the original proposal, the costs, training and special facilities needed for handling the radioactive isotopes were mentioned as major problems of RFLP analysis and it was proposed to investigate the use of non-radioactive probes. As well, the complete RFLP procedure requires large amounts of DNA (although blots can be reused) and a considerable amount of time. On the other hand, the RAPD method requires the equipment ($6,000-$15,000) for doing PCR reactions, and the large sets of random primers needed for the initial screening. In fact, it was very fortunate that, at the time we wished to test some RAPD methodology, some primers were available in small amounts and at a nominal cost ($250) from the University of British Columbia. If large numbers of different primers had to be made, it would be very expensive. As well, with PCR, care must be taken in carrying out reactions, as small contaminants can obscure the results. The technical advantage of the RAPD method is that the products of the PCR reactions can be separated directly on agarose gels and visualized or photographed under uv light after ethidium bromide staining. Thus, there is no need for blotting DNA to membranes, preparation of probes, hybridization methodology or long exposures of X-ray film. Another difference in the application of the technology lies in the requirements for using the techniques and the extent to which the technology is protectable and portable. In order for someone to use RFLP methodology, the appropriate restriction endonuclease must be known and the specific DNA used for probe preparation must be available, usually cloned into a plasmid vector. For the RAPD method, only the sequence of the oligonucleotide primer is needed, as the primer itself can be synthesized in many labs or by commercial DNA synthesizing laboratories.

There is also a difference in the amount of information which can be obtained from the two methods. RFLP analysis produces, in most cases, DNA fragments of different lengths, hybridizing to the same probe. Their differences are caused by sequence differences at restriction sites or deletions between them, although sometimes null alleles are detected (see Report #1). With two such visible alleles, the analysis of a hybridized blot (Figure 5), can identify both of the homozygous and the heterozygous (both bands present) genotypes. This is not usually the case with RAPD markers. In no case did the PCR reaction produce two bands which segregated as different alleles. Rather, all were scored as plus/minus with a 3:1 ratio of the presence of the band to its absence. Because of this, it was not possible to specify the genotype of those plants containing the band, only the null state showed unequivocally a homozygote. If one is using RAPD markers to examine quantitatively controlled traits which have additive gene action, the inability to distinguish the homozygous from the hemizygous presence of the marker condition may make it difficult to establish the genetic linkage of the marker or to carry out efficient selection using the marker.

Recently, PCR methodology is being applied in the quantitative determination of nucleic acids and a similar approach could help with this problem. Observation of the ethidium-stained gels with products from the PCR amplification of DNA from F_2 plants (Figure 6) suggests that there may indeed be differences in the amount of PCR-amplified DNA from different plants, although the interpretation is complicated by the differences in gel loading. To examine this in more detail, the photographic negatives of a gel separating the PCR products from primer RC41 and DNA from parent and F_2 plants, were scanned on a Molecular Dynamics densitometer. Three integration lines were drawn across all the lanes as follows: a - one through a polymorphic band showing segregation b - one between bands, to estimate the lane background c - one through a distinct band common to all samples, to be used in correcting for sample loading. The areas under the three lines were determined for all plants and the relative amount of DNA in each of the polymorphic bands was estimated by the following formula:

$$\text{Relative DNA} = \frac{\text{Area (a)} - \text{Area (b)}}{\text{Area (c)}}$$

Data from 37 F_2 samples were obtained and a χ^2 analysis showed that the distribution was significantly ($P<0.05$) different from normal. The samples could be allocated to three groups based on their relative DNA as in Table 6. Despite the small sample number, the results are interesting in that the estimated DNA content of Group 1 was not different from zero, and the average for Group 3 was twice that found in Group 2, as would be expected if Group 2 consisted of hemizygous genotypes. Finally the numbers in these groups were not significantly different from a 1:2:1 ratio expected in

Table 6. Distribution of F_2 plants on the basis of estimated DNA content of RAPD polymorphic bands.

CHARACTERISTIC	GROUP 1	GROUP 2	GROUP 3
NUMBER OF PLANTS	10	19	8
MEAN RELATIVE DNA CONTENT	0.12	0.79	1.45
STANDARD DEVIATION	0.10	0.24	0.22

a single gene segregation. The distribution of RAPD markers in F_2 plants was identical when primers UBC521 and UBC573 were compared. Moreover, both were significantly ($P<0.01$) correlated with the results from the RFLP analysis with BglII enzyme and LRF15 probe when the RFLP data were scored as 0, 0.5 (heterozygous) and 1.0 as compared to 0 and 1 for the RAPD's. Relative DNA contents were estimated for the polymorphic bands in both RAPD gels, as described above, and when the F_2 samples were grouped according to the RFLP data, the mean DNA contents (both primers) for the 3 groups were 0.23, 0.49 and 0.66. Although it is not known how closely the RAPD markers are linked to this RFLP marker, these results support the idea that the amount of DNA in specific PCR products may reflect the relative amount of DNA in the original sample. This possibility is probably worth pursuing as the ability to discriminate hemizygous from homozygous conditions could add considerable power to RAPD technology.

The results from four plants of each parent and 40 F_2 plants with all RFLP and RAPD markers are presented in Appendix 5 ASCOCHYTA AND MARKERS, along with the scoring for the presence of *Ascochyta* in seed. Because specific data from either RFLP or RAPD methods are not available for all of the individual plants, direct comparisons of markers with each other and disease scores are difficult. A second set of data was prepared to provide a complete matrix for direct comparisons. This set (Appendix 5 CORRELATION GRID) contains results with all markers for 28 individual F_2 plants. RAPD markers, scored only as 0 or 1 (absence or presence), do not provide a full identification of the genotype. However, the scoring in this table as 1 = the Eston phenotype and 0 = the ILL5588 phenotype for both RFLP and RAPD markers, with heterozygotes from RFLP markers scored as 0.5, makes it possible to compare markers with each other and with the *Ascochyta* data (Eston having higher values than ILL5588 for both disease and markers). A simple correlation coefficient can be used to examine the relationships among the markers and disease response (Appendix 5 CORRELATION COEFFICIENTS). Among the 20 markers, several groupings were found.

1. <u>BclI/LRF5, BglII/LRF5, U535</u>: The two RFLP markers gave identical results, not only in these 28 plants, but as well, in all plants for which data were available. As these use the same probe, it is expected that a deletion was involved rather than a difference in restriction site. These markers were significantly ($P<0.01$) correlated with the pattern of RAPD's from U535. While not significant, the correlations between these and disease were positive.

2. <u>BglII/LRF15, U540.2, U540.3</u>: These formed a relatively weak group, but all showed negative association with disease.

3. <u>BglII/LRF15, U521, U573, U599</u>: Markers from U521 and U573 produced identical results in all genotypes examined and the other two were correlated with them. However, while U521 and U573 were weakly associated with the presence of disease , BglII/LRF15 and U599 were negatively related to it.

4. <u>U516.2, U519.1, U585, U598</u>: These were generally correlated with each other, but all were generally negatively associated with the disease trait.

From the examination of all these data, a number of markers were selected on the basis of general association with the disease resistance. In cases where two identical markers were selected, only one was used for further study. The data were coded in a different way in an attempt to reflect the theoretical number of copies of *Ascochyta* resistance genes which might be contributed from the parents to the progeny, if individual genes were linked to individual markers. Thus for RFLP markers, 0 indicated the allele from Eston, 1 indicated the heterozygous condition (1 copy) and 2 indicated the allele associated

with ILL5588. For RAPD markers, the situation was more complicated. If parent genotype ILL5588 showed the presence of the polymorphic band, then its absence in an F_2 plant would indicate no resistance genes (score = 0) and its presence could be either 1 or 2 copies (score = 1.5). On the other hand, if ILL5588 parent plants did not exhibit the band, then F_2 plants lacking the band would also be homozygous for the ILL5588 gene (score = 2) and those with the band might have either 0 or 1 copy from ILL5588 (score = 0.5). After scoring each genotype for markers BclI/LRF5, BglII/LRF15, RC41, U516, U521, and U535, additional combined markers were created by adding the scores of individual pairs or groups of three. In no case was there a significant correlation between the individual or combined marker scores and the *Ascochyta* scores when all individual plants were considered. However, to minimize the effects of uncertainties with RAPD markers, the genotypes for which complete data were available (33) were separated into 3 groups of 11 on the basis of their *Ascochyta* data. These, along with the group means for both marker scores and *Ascochyta* prevalence, are presented in Appendix 5. RFLP AND RAPD COMBINATIONS. The correlation coefficients for the markers and combinations of markers, which showed the strongest association with *Ascochyta* resistance, are presented in Table 7. These results indicate 4 markers, which alone or in combination, might be

Table 7. Correlations between estimated number of marker genes or combinations of marker genes and the presence of *Ascochyta* in seed of F_2 plants

NUMBER OF MARKERS	MARKER NAMES Correlation Coefficients (* P<0.05)					
1	BclI/LRF5	RC41	U521	U535		
	-0.91	-0.65	-0.65	-0.999*		
2	BclI/LRF5			RC41		U521
	RC41	U521	U535	U521	U535	U535
	-0.997*	-0.999*	-0.978	-0.64	-.94	-0.91
3	BclI/LRF5			RC41		
	RC41	RC41	U521	U521		
	U521	U535	U535	U535		
	-0.97	-0.999*	-0.998*	-0.86		

associated with the major genes controlling the resistance trait from the ILL5588 genotype. It must also be pointed out that BglII/LRF5 gave the same results as BclI/LRF5 and U573 was identical to U521. Thus a total of 6 markers have been identified and should be tested in more extensive experimentation.

7. Assessment of Project Results

A molecular marker can be very useful once it has been established that its pattern, whether presence/absence or different bands for the two parents, has a high probability of segregating along with the plant trait of interest. Then, selection for the trait can be indirectly accomplished by selecting for the molecular marker, which can be detected in any tissue, at any stage of development and in plants grown under any environmental conditions that are not lethal. This project undertook to develop such molecular markers for the *Ascochyta* resistance character which is being used in Saskatchewan lentil breeding. In some ways this was a good test of the technology . This genetic resistance to *Ascochyta*

appears to be associated with two major dominant genes and a less important recessive gene. The very reason for wanting molecular markers is that assessment of plant response to fungal pathogens in the selection phase of breeding programs is very difficult, time consuming, labor intensive and expensive. Thus the same problems face a researcher in trying to establish the relationship between markers and disease resistance genes. The only germplasm, of known genetic makeup, with respect to the genes involved in the resistance trait, were the parent lines. Linkage relationships had to be established on the basis of correlations between markers and disease in individual F_2 plants. Even in a disease nursery, lack of symptoms can be caused by either genetic resistance characteristics of the plant or by the effects of the microenvironment on a particular plant. Thus there is always some question about the actual genotype of the individual plants. Added to these difficulties is the added uncertainty associated with distinguishing homozygous from hemizygous genotypes when RAPD markers are used. Despite these problems, four independent markers were found to be closely associated with the segregation of the disease resistance trait and some general comments can be made about the different parts of the work.

1. The number of RFLP markers which could be used was disappointing. The four that were used (two being the same) were all based on probes obtained from an outside source, but they showed clearly distinguishable bands and good segregation ratios in our F_2 population.

2. The RAPD methodology provided a very large number of potential markers with variability between lentil genotypes as well as useful markers with consistent differences between resistant and susceptible genotypes and correct segregation ratios in the F_2 plants. Of the final markers studied, 16 were developed by this method.

3. RAPD markers are more easily found within *Lens culinaris L.* and are more easily, safely and cheaply produced than are RFLP markers. On the other hand, the inability to distinguish homozygous from hemizygous genotypes using RAPD markers is a serious drawback to their use. The preliminary indications that quantitative differences in PCR products could be used to distinguish between them should be pursued.

4. Although no assessment of vegetative *Ascochyta* disease symptoms could be made on the parent and F_2 populations in the disease nursery, the assessment of disease in the individual plants on the basis of the pathogen presence in the seeds was very successful considering all of the uncertainties in disease rating discussed above. The data in replicate seed sets were very reproducible, the distribution of the individual plants on the basis of the assessment was not incompatible with current understanding of the genetic system and this evaluation system would have removed the complication of a third disease resistance gene which is not expressed late in the season.

5. Methodology was developed which would make it possible to screen large numbers of individual plants. DNA which is of sufficiently good quality and quantity for 2 RFLP or 50 RAPD samples can be extracted from as little as 50 mg of leaf tissue, so that plants can be sampled early in development. It is estimated that 50 samples per day could be collected from the field and stored or extracted at a rate of 30 per day.

6. Twenty genetic markers which distinguish between Eston and ILL5588 genotypes were identified in this work. These are all readily visualized and had segregation ratios appropriate to single gene segregation. Groupings were apparent among these markers, in some cases probably due to the detection of the same chromosome region and in others to greater or lesser degrees of genetic linkage. On the basis of the relationships

between markers and disease severity, six markers were selected as having the potential to indicate the existence of the *Ascochyta* resistance genes in ILL5588.

8. Application of Results

In the original proposal it was pointed out that the project would be working in collaboration with Saskatchewan lentil improvement programs. Indeed, Dr. A.E. Slinkard of the Crop Development Centre was designated as a collaborator and supplied seed of not only the parent lines, but of advanced breeding lines which were used in the initial screening of RFLP markers. Moreover, for the *Ascochyta* assessment work during the summer of 1991, Dr. Slinkard grew and inoculated our plant populations and Drs. Morrall and Peterson of the University of Saskatchewan's Biology department, the pathologists associated with lentil work in Saskatchewan, assisted with the evaluation of disease. Thus there has been continuous contact between the project and researchers involved in applied lentil programs. From the results, certain recommendations can be made about the possible applications which might be made of this work.

The six markers, identified in this study as most closely associated with *Ascochyta* resistance genes, should be tested in one or more larger populations of plant material being examined for the resistance trait. Preliminary discussions with Dr. Slinkard have indicated that such tests will in fact be planted out during the summer of 1992 and that with a moderate cost and effort, the marker distributions in these plants could be screened. Already, large batches of the primers needed for all of the RAPD markers and large plasmid preparations for RFLP markers have been prepared. Because, as it turned out, the only probe which produced an RFLP marker related to the *Ascochyta* resistance phenotype was obtained from Dr. Muehlbauer, it cannot be released to others without his permission. The primers used to generate all of the RAPD markers were purchased outright and the exact sequences of those producing all of the RAPD markers which distinguished cv Eston from the ILL5588 are listed in this report. Permission of the ADF would be requested for any further use of these primers for use in *Ascochyta* resistance work.

In a more general sense, the work has revealed the difficulties associated with the use of RAPD technology for quantitative characteristics such as disease resistance. It has also raised the possibility that some modifications to the procedures used in the development of RAPD markers could overcome this problem and make it possible to obtain similar data as can be achieved with RFLP markers. Theoretical considerations discussed in this report suggest that genotypes homozygous for the presence of the marker band may produce a greater quantity of PCR product than do the hemizygous genotypes, and some preliminary densitometer analysis of the data from some RAPD gels produced results which tend to support the idea. If this could be established as a general principle, then the usefulness of RAPD technology could be considerably enhanced. Development and testing of this methodology could be more easily done using the RAPD markers and genetic material developed during this project. It is understood that the use of any such material arising out of the work would require the expressed permission of the ADF.

Publications have not yet been prepared from this work; however, now that it is apparent that many of the objectives have been attained, such publication would be desirable. Due notification to ADF and requests for permission will be made as such manuscripts are prepared.

NON-TECHNICAL SUMMARY

Recently there has been considerable publicity about the use of new genetic markers which can directly detect differences in the DNA molecules that make up genes. These markers have been used to create "genetic fingerprints" which, because they are unique to individuals, can be used for identification in court cases. As well, when one of these markers is almost always associated with a particular genetic disorder in people, it must be situated on one of the chromosomes very close to the gene which is different from the normal type. Using new molecular biology techniques and such markers, the actual genes responsible for some diseases have been found and the small difference causing them identified. As is often the case, these methods, initially developed for other purposes are now beginning to be used in the study of crop plant species and there is hope that they can help plant breeders identify genetic relationships between plants, indirectly select for important traits that are difficult or expensive to measure and identify locations on the chromosomes where important genes are found.

Whenever new sources of genetic resistance to a particular plant pathogen are found, a major varietal improvement follows as the gene becomes a part of all new varieties. However, the process of creating these new varieties with both the new disease resistance and the established agronomic and quality traits is time consuming. This is especially the case with genes for resistance to fungal pathogens, because at every stage of the process, the material has to be tested for its ability to resist the fungal attack. However, fungal infections are unpredictable and even in disease nurseries, no disease may develop on susceptible plants and a selection season may be lost. It has been suggested that these new DNA-based genetic markers may help to overcome these problems because their detection is completely independent of the environment. Thus, if a marker can be found that is close to the gene making the plant resistant, the two will tend to stay together whenever new plants are produced. If the marker is easily detected, it can identify those plants that have the resistance trait, without going through the expensive and difficult infection and evaluation of the plant disease. This research project was based on the recognition that the new genetic resistance to *Ascochyta* blight found by Dr. A.E. Slinkard, a lentil breeder at the Crop Development Centre, would provide a good opportunity to search for such molecular markers and to examine their relationship to the genes controlling the resistance.

Two kinds of markers, known as RFLP and RAPD were used and both produce easily seen bands which indicate the presence of a specific sequence in the DNA molecules making up the chromosomes. Also in both cases, large numbers of marker bands can be produced, and the first real difficulty is to find those that are differ between plants with and without the disease resistance trait. In this work, from more than 500 possible RFLP markers examined, only 4 had potential to be useful and from 140 primers, 22 RAPD markers looked promising. The second step is to select those that are located close to the disease gene(s), and the only way to do this is to examine both the markers and the disease response of individual plants from the F_2 population of a cross between resistant and susceptible parents. In this generation, the genes conferring resistance will be randomly distributed to some plants but not others and if a marker is closely linked to the resistance genes, then plants with the marker should have little disease. In the summer of 1991, parent and F_2 progeny plants were grown in the Crop Development Centre's disease nursery . Leaf tissues were collected during the season and DNA was extracted from them for RFLP and RAPD marker detection. At the end of the season, seeds were collected from each plant and analyzed for the presence of *Ascochyta*. The data from four plants of each parent type and 40 F_2 plants were used to examine the relationships between markers and the presence of disease. One RFLP and three RAPD markers showed similarity in their

distribution among the progeny plants to the apparent protection from the disease and at least two combinations of the RFLP marker with RAPD markers showed significant correlations between the estimated number of marker genes and the resistance to disease. These markers should be tested in large-scale trials.

In general, the results show that for lentils, RAPD markers are more easily found than RFLP markers. Moreover, they are also safer and easier to work with than RFLP markers which require transfer of DNA from gels to blotting membranes, hybridization techniques and radioactive isotopes. On the other hand, while RAPD markers show the same result for either one or two copies of the marker gene, RFLP markers show a different pattern for the two. Thus, a plant breeder working with RFLP markers can distinguish between homozygous and hemizygous genotypes and therefore select plants which will produce only pure breeding progeny. This increased precision of RFLP markers might outweigh the technical advantages of RAPD markers, but some preliminary work done during this experimentation suggests that it may be possible to modify the RAPD procedures and recover the same information as is now available only from RFLP markers.

PERSONNEL

The following personnel were involved in the project:

Supported by ADF

Dr. Om Rajora	9/4/90 to 30/5/91	2100 h
Mr. Colin Kindrachuk	1/4/90 to 31/3/92	3675 h
Mr. Jiyue Liu	1/6/91 to 31/3/92	1510 h

Supported by NRC

Dr. John Mahon	1/2/90 to 31/3/92	479 h
Dr. L. Pelcher	13/1/92 to 1/4/92	94 h

As discussed in previous reports, the time required for administering and directing the research involved in this contract required approximately 5% of Dr. Mahon's time. Indeed, the total expenditure of time by Dr. Mahon on the project was approximately this until the departure of Dr. Rajora, after which time it increased as more scientific management was required. Dr. Pelcher's involvement was initiated when the decision was made to test RAPD markers as well as RFLP markers. During that 4 month period, he carried out the PCR reactions needed for the work. The time expended by these PBI personnel on this work has not been charged to ADF. Rather, the information is presented for the following reasons:

a) to demonstrate how funding by ADF can stimulate investment in research activity, of interest to Saskatchewan agriculture, beyond that directly supported by the contract itself

b) because the contract stipulates that ownership in any intellectual property arising from the work is to be determined on the basis of direct operating costs and the salaries for these hours would represent such a direct expenditure on the project by NRC.

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APPENDIX 1.

INCIDENCE OF ASCOCHYTA IN LENTIL SEED

Seed was collected from individual plants growing in the Crop Development Centre's disease nursery at the end of the 1991 growing season and percent of seeds containing *Ascochyta* in 10-seed lots was determined for each as described. Genotype 1 is Eston and E1 to E4 are the plants used as representative of Eston for comparisons of RFLP and RAPD markers in the F_2 progeny. Genotype 3 is ILL5588 and I1 to I4 are the plants used as representative of ILL5588. Plants in genotype 4 are F_2 progeny from an Eston x ILL5588 F_1 plant and genotype 5 plants are from an ILL5588 x Eston F_1 plant.

APPENDIX 1. INCIDENCE OF ASCOCHYTA IN LENTIL SEED

			Percent Seeds with Ascochyta Infection															
							Plate Number									Mean	S.D.	Ln (X+1)
Anal#	Bl	Gen	Pl	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12			
ESTON																		
E1	1	1	3	60	50	70	90	40	50	70	40					58.8	17.3	4.09
	1	1	4	60	60	20	50	20	30							40.0	19.0	3.71
	1	1	5	60	20	0	10	100	75							44.2	40.1	3.81
	1	1	6	30	10	0	20	10	10	0	30	10	40	50	0	17.5	16.6	2.92
	1	1	7	0	0	0	0	30	0	50	10	100	100	100	100	40.8	46.2	3.73
	1	1	10	0	0	0	30	50	30	30	80	40	10	90	90	37.5	33.9	3.65
	1	1	11	30	50	30	20	30	40	50	20	60	70			40.0	17.0	3.71
E2	1	1	12	70	60	10	70	100	30	50	90	40	40	20	40	51.7	27.2	3.96
E3	1	1	13	70	40	60	30	80	70	0	100	80	80	0	100	59.2	34.5	4.10
E4	1	1	14	50	60	40	50	20	10	30	30	60	60	70	83	46.9	21.5	3.87
ILL5588																		
	1	3	1	0	0	0	0	0	0	0	0					0.0	0.0	0.00
	1	3	3	0	0	0	0	10	0							1.7	4.1	0.98
I1	1	3	4	0	10	0	0	0	0	0	0					1.3	3.5	0.81
	1	3	6	0	0	0	0	0	0	0	0					0.0	0.0	0.00
I2	1	3	10	0	0	0	0	0	0	0	20	0	0			2.0	6.3	1.10
	2	3	2	0	0	0		0	0	0	0					0.0	0.0	0.00
I3	2	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
	2	3	6	0	0	0	10									2.5	5.0	1.25
I4	2	3	7	0	0	0	0	0	10	0	0	0	0	0	0	0.8	2.9	0.61
	2	3	8	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
	2	3	9	0	0	0	0	0	0	0						0.0	0.0	0.00
	2	3	12	0	0	0	0	0								0.0	0.0	0.00

APPENDIX 1. INCIDENCE OF ASCOCHYTA IN LENTIL SEED

	Bl	Gen	Pl	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	MEAN	S.D.	Ln(X+1)
F2 ESTON x ILL5588, ILL5588 x ESTON																		
F1	1	4	2	0	0	0	10	0	0	0	0	0	10	0	0	1.7	3.9	0.98
F2	1	4	3	0	10	10	0	20	20	0	20	70	90	70	90	33.3	35.8	3.54
F3	1	4	5	0	0	0	0	0	0	0	0	0	10	0	0	0.8	2.9	0.61
F4	1	4	6	10	0	10	20	20	20	20						14.3	7.9	2.73
F5	1	4	8	10	0	0	10	0	0	20	10	40	20			11.0	12.9	2.48
F6	1	4	9	10	0	0	0	0	0	0	10	10	0			3.0	4.8	1.39
F7	1	4	10	100	100	100	80	70	90	100	40	80	70			83.0	19.5	4.43
F8	1	4	11		70	50	70	30	20	10	60	10	90	80	70	50.9	28.8	3.95
F9	1	4	12	50	0	0	0	0	0	0	10	0	0	0	0	5.0	14.5	1.79
F10	1	4	13	80	100	100	100	100								96.0	8.9	4.57
F11	1	4	14	0	0	0	0	0	0	0	0	0	0	10	0	0.8	2.9	0.61
F12	1	4	15	0	10	0	0	0	0	0	0					1.3	3.5	0.81
F13	1	4	17	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
F14	2	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
F15	2	4	2	0	20	0	10	0	0	0	10	0	0			4.0	7.0	1.61
F16	2	4	4	0	0	0	0	0	0	0	0	0	0			0.0	0.0	0.00
F17	2	4	6	0	0	0	0	20	0	10	0	0	0	0	0	2.5	6.2	1.25
F18	2	4	7	0	20	0	0	10		10	50					12.9	18.0	2.63
F19	2	4	10	0	0	0	10	0	0	10	0	0	0	0	0	1.7	3.9	0.98
F20	2	4	14	0	0	0	0	0	0	0	0	0	20	20	30	5.8	10.8	1.92
F21	2	4	15	0	0	0	0	0	0	0	0					0.0	0.0	0.00
F22	2	4	16	0	0	0	0	0	10	0	0	10	0	0	0	1.7	3.9	0.98
F23	2	4	17	70	30	60	50	50	30	0	60	70				46.7	22.9	3.86
F24	2	4	18	90	70	70	60	50	50	80	70	50	0	20	20	52.5	27.0	3.98
F25	2	4	20	0	0	0	0	10	0	0	0	0	0	0	10	1.7	3.9	0.98
F26	2	4	22	10	10	10	0	10	30	0						10.0	10.0	2.40
F27	2	4	23	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
F28	1	5	4	0	0	0	0	0	0	0	0	0	0	0		0.0	0.0	0.00
F29	1	5	7	0	0	0	0	10	10	0	10					3.8	5.2	1.56
F30	1	5	8	0	0	0	10	10	0	0	0	10	0	10	0	3.3	4.9	1.47
F31	1	5	10	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
F32	1	5	11	0	0	0	0	10	0	0	0	0	0	0	0	0.8	2.9	0.61
F33	1	5	13	0	0	0	0	0	10	0	0					1.3	3.5	0.81
F34	2	5	2	0	0	0	0	0	0	10	0	0	10	0	0	1.7	3.9	0.98
F35	2	5	7	10	0	0	10	0	0	30	10	0	0	0	0	5.0	9.0	1.79
F36	2	5	12	0	0	0	0	0	0	0	10	0	0	0	0	0.8	2.9	0.61
F37	2	5	13	0	10	0	0	10	0	10	10	0	0	0	0	3.3	4.9	1.47
F38	2	5	20	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00
F39	2	5	22	10	0	0	10	30	0	10	10	0	10	10	10	8.3	8.3	2.23
F40	2	5	23	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.00

APPENDIX 2.

EXTRACTION OF PLANT DNA

Several methods of extracting DNA from plant material were used for different purposes. Four methods are presented.

Large Scale CTAB Method:

This was the main method used for material grown indoors, when large amounts of DNA were required for methodology development of routine analysis of the same material.

Mini Methods:

Three different methods were tried for rapid DNA preparation from small samples of plant material, as would be used in routine analysis of individual plants in field trials. They produced sufficient DNA for two Southern blots. These methods were modified from methods developed for larger samples and were called:

Mini CTAB
Mini Dellaporta
Mini Phenol/Chloroform

APPENDIX 2. EXTRACTION OF PLANT DNA

Large Scale CTAB Method (Modified from Murray and Thompson, 1980)

2 X CTAB (Hexadecyl trimethylammonium bromide) Extraction Buffer
(Keep at 60C to prevent precipitation of CTAB)

Tris HCl, pH 8	.125 M
NaCl	1.75 M
EDTA	.0156 M
CTAB	2.5%
2-Mercaptoethanol	.25%

1. Chill mortars and pestles in -20C
2. Select 1 - 1.5 g of young leaves (wash and dry)
3. Grind leaves to a fine powder in liquid nitrogen and transfer powder to 50 ml Teflon Oakridge tubes containing 2 X CTAB extraction buffer.
4. Incubate at 60C for 60 min. with occasional shaking
5. Extract once with 15 mls chloroform/octanol (24/1).
6. Centrifuge at 9,000 rpm at 15C for 15 min
7. Remove aqueous top layer with pasteur pipette to clean test tube.
8. Add 2/3 volume cold isopropanol and mix gently to precipitate white strands of DNA. If no precipitation occurs, place at -20C for 30 min.
9. Transfer DNA to 5 mls 76% ethanol, 10mM ammonium acetate and leave for 20 min at room temperature.
10. Transfer DNA to microfuge tubes and dry in a vacuum centrifuge.
11. Resuspend pellet in of 500 μl TE buffer.

Mini CTAB DNA Extraction Method

2 X CTAB Buffer

Tris HCl (pH 8.0)	100 mM
EDTA	25 mM
NaCll	1.4 M
CTAB	2 %

1. Place 25 - 100 mg young leaf material into a 1.5 ml Eppendorf tube on dry ice. These samples can be extracted immediately or stored at -70C for later extraction.
2. Grind to a fine powder with a pestle.
3. Add 250 μl of extraction buffer (preheated to 60C) and grind at room temperature with the pestle.
4. Wash the pestle with an additional 250 μl extraction buffer. Vortex briefly.
5. Incubate in 60C water bath for 40 min.
6. Extract with 500 μl chloroform / isoamyl alcohol (24:1)
7. Vortex 5 min, Centrifuge at 15000 rpm for 5 min.
9. Transfer aqueous phase to a new tube
10. Add 400 μl isopropanol (cooled to -20C)
11. Centrifuge 15 min..
12. Decant. Wash with 500 μl 80% Ethanol. Centrifuge 5 min. Repeat and dry.
13. Take up in 20 μl TE buffer.

APPENDIX 2. EXTRACTION OF PLANT DNA

Mini Dellaporta Extraction Method (Modified from Dellaporta et al., 1985)

Extraction Buffer

Tris HCl, pH 8.0	100 mM
EDTA	50 mM
NaCl	.5 M
2-Mercaptoethanol	10 mM

1. Place 25 - 100 mg young leaf material in 1.5 ml Eppendorf tube on dry ice. These samples can be extracted immediately or stored at -70C for later extraction.
2. Grind to powder with pestle.
3. Add 250 µl extraction buffer and grind on ice.
4. Add 30 µl 20% SDS. Vortex.
5. Incubate 10 min. at 65C.
6. Add 150µl 5 M potassium acetate. Vortex
7. Incubate on ice 30 min.
8. Centrifuge at 5C for 20 min.
9. Decant supernatant carefully into a new tube (if necessary, spin and decant again)
10. Extract with 600 µl of phenol / chloroform (1/1)
11. Vortex 5 min. Centrifuge 5 min.. Transfer aqueous upper layer to a clean tube.
12. Wash with 500 µl of ether twice.
13. Add 400 µl of isopropanol. Vortex. Place at -20°C for 30 min.
14. Centrifuge 15 min..
15. Decant. Wash with 500µl 80% Ethanol. Centrifuge 5 min. Repeat and dry.
16. Take up in 20 µl TE buffer.

Mini Phenol / Chloroform DNA Extraction Method

Extraction Buffer

Tris HCl (pH 8.0)	100 mM
EDTA	5 mM
NaCl	100 mM
SDS	1 %

1. Place 25 - 100 mg young leaf material in 1.5 ml Eppendorf tube on dry ice. These samples can be extracted immediately or stored at -70C for later extraction.
2. Grind to powder with pestle.
3. Add 400 µl phenol/chloroform (1/1) and grind with pestle on ice
4. Wash pestle with 600 µl extraction buffer.
5. Vortex for 5 min.
6. Centrifuge at 15,000 rpm for 5 min.
7. Transfer aqueous layer to a new tube.
8. Wash twice with 500 µl of diethyl ether
9. Add 400µl isopropanol. Place at -20 C for 30 min.
10. Centrifuge 15 min..
11. Decant. Wash with 500 µl 80% ethanol. Centrifuge 5 min. Repeat and dry.
12. Take up in 20 µl TE buffer.

APPENDIX 3. SOUTHERN HYBRIDIZATIONS FOR RFLP ANALYSIS

RESTRICTION DIGESTS

For all restriction digests, restriction endonucleases from Boehringer Mannheim were used along with the reaction buffers and conditions recommended by the supplier. For most routine analyses, the amount of DNA in each sample was estimated either by visual comparison of ethidium bromide staining on gels or by ultraviolet absorption (A_{260}) and volumes required for approximately 10 μg of DNA were added to each reaction mixture as follows:

Reaction buffer x 10 (Boehringer Mannheim)	2 μl
Sample	5 to 15 μl
Restriction Enzyme	2 μl
water	11 to 1μl
TOTAL	20 μl

Reactions were carried out for 4 to 5 hours, usually at 37C, unless another temperature was recommended by the supplier of the restriction enzyme. After this incubation, DNAase-free ribonuclease (1 μl of 10 mg mL^{-1}) was added and the reaction allowed to proceed for another 45 min. The reaction was stopped and the samples prepared for loading onto gels by adding 5μl of loading buffer (0.125 g bromophenol blue in 30% glycerol, 25 mM EDTA).

AGAROSE GEL ELECTROPHORESIS

For blots used to examine all combinations of 8 restriction endonucleases (BamHI, BclI, BglII, DraI, EcoRI, HindIII, KpnI, ScaI) with 7 lentil genotypes, or the 8 parent plants and 40 F_2 plants digested with a common restriction enzyme to determine the segregation of RFLP markers, a 300 ml gel was cast using 0.8% agarose LE (SeaKem Inc.) with 150 μg of ethidium bromide in TBE buffer (Maniatis et al., 1982. These were combined and heated in a microwave oven to dissolve the agarose and the solution was poured into a 24 x 20 cm casting bridge with two combs, each having 30 wells for sample loading. After the gel cooled, it was placed in an IBI Horizontal Electrophoresis unit and just covered with TBE buffer. Samples and standards of HindIII- cut λ phage (20 μl) were layered under the TBE buffer, the power was turned on and the gel was run at 36-40 V for 14 to 16 hours, after which it was removed from the apparatus, photographed under uv light and, for ease in handling, the upper (A) and lower (B) halves of the gel were separated by cutting along the top of the second set of wells. Thereafter, the two gels were handled separately.

SOUTHERN BLOTTING

As discussed in Report #2, a number of methods were tested for the preparation of Southerns and the method eventually used for most of the work is as follows

1. Wash gel with gentle rocking in 0.25M HCl until dye turns from blue to yellow.

APPENDIX 3.

SOUTHERN HYBRIDIZATIONS FOR RFLP ANALYSIS

Several methods were tested, and these are the final best methods for all of the steps leading to the resolution of RFLP markers.

2. Wash gel with gentle rocking in Alkaline Transfer Solution for 30 min. Dye should return to blue colour.

Alkalai Transfer Solution:	NaOH	0.4 M
	NaCl	0.6 M

3. Float Hybond N+ membrane (DuPont Co.) on Alkaline Transfer Solution for 10 min at room temperature.

4. Construct blot in pyrex tray as: 7 sheets of blotting paper
gel
Hybond N+ membane
3 sheets of filter paper
2 cm thick layer of paper towels
200 g weight

5. Add Alkaline Transfer Solution to the pyrex tray and replenish when required for a 3 to 5 hr period at room temperature.

6. Disassemble the blot, remove membrane and rinse for 10 min in 0.2M Tris/HCl +2 x SSC (Maniatis et al., 1982)

7. Dry membrane between layers of blotting paper and bake at 85C for 2 h.

PREPARATION OF DNA FOR PROBES

DNA for producing radioactively labeled probes came from either Dr. Muehlbauer in plasmid cloning vectors or from the cDNA library produced by Clontech Laboratories Inc. from RNA extracted at PBI from Laird lentils. In this library, cDNA fragments were cloned into the EcoRI site of a λgt11 cloning vector as was described in Report #2. The library was plated out and individual plaques stored in 1 ml of SM medium with a drop of chloroform (Maniatis, 1982) for future use. The protocol for isolating λDNA from these clones is presented is as follows:

1. For each of 4 plates, add 100 μl of an overnight culture of Y1090 plating bacteria and 10 μl of a single plaque λgt11 isolate to 4 ml of cooled 0.7% agarose, Mix quickly and pour over plates of NZCYM medium containing 30 μg ml^{-1}.

2. Incubate at 37C overnight.

3. Scrape the top agar of plates with clearly visible plaques into a 50 ml centrifuge tube containing 15 ml of λ dilution buffer (10 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA).

4. Shake (300 rpm) at room temperature for 2 h on rotary shaker.

5. Centrifuge at 16,000 x g for 30 min at 4C.

6. Decant supernatant into clean tube and repeat the centrifugation.

7. Decant supernatant into another clean centrifuge tube and add 20 μl of RNAase (10 μg μl^{-1}) and 10 μl of DNAase (10 u μl^{-1}). Incubate at 37C for 40 min.

8. Add equal volume of PEGS solution (20% polyethylene glycol 8000, 2 M NaCl in λ dilution buffer) mix and stand on ice 30 min.

9. Centrifuge 10,000 x g for 20 min.

10. Decant supernatant, drain pellet completely, dissolve pellet in 500 μl of TEN buffer and transfer to microfuge tube.

11. Extract with 500 μl of phenol/chloroform, vortex for 5 min, centrifuge 5 min.

12. Transfer aqueous layer to new tube, add 500 μl chloroform, vortex for 5 min and centrifuge for 5 min.

13. Transfer aqueous layer to new tube, add 500 μl isopropanol, stand at -20C for 30 min.

14. Centrifuge, wash pellet twice with 80% ethanol and dry

15. Take up pellet in 100 μl TE buffer.

The DNA found in these preparations contained cDNA inserts of varying sizes, located between two EcoRI restriction sites. In order to produce probes of sufficiently high specific radioactivity to produce a clear signal when hybridized to single copy genes in the lentil genomic DNA bound to the Southern blots, the cDNA had to be separated from the phage DNA. The following three methods were used to produce large quantities of relatively pure insert DNA.

1. A sample of the appropriate λ DNA was digested with EcoRI, treated with RNAase and run on a preparative 1% low melting point agarose gel (SeaPlaque). The part of the gel corresponding to the insert was cut out and the DNA was extracted from the gel and cleaned on a Quiagen Tip 5 according to the manufacturers instructions.

2. A PCR reaction was carried out using primers homologous to regions of the λ gt11that spanned the EcoRI site into which the inserts had been cloned. From the location of the primer annealing sites, it could be estimated that the PCR product would exceed the length of the insert by 163 bp. Estimates from gels showed the lengths of the PCR products from 8 clones to exceed the lengths of the EcoRI fragments of the same clones by an average of 168 ± 18 bp.

3. Because the insert comprises such a small proportion of the λgt11 DNA (1 kb fragment $\approx$ 2.5%), recovery of large amounts of insert were difficult. A third approach to recovering inserts was to transfer the inserts to a plasmid. To do this the library samples were plated, but rather than selecting individual clones, the library was harvested as a single sample and a bulk DNA extraction was carried out. This DNA was digested with EcoRI and separated on a 1% agarose gel to produce a large λ band(s) and a smear representing inserts of various sizes. Two or three size classes of inserts were run onto separate NA45 (Schleicher and Schuell) papers and the DNA was removed with a high salt buffer. These heterogeneous inserts were ligated into the EcoRI site of a pT7T318U plasmid which was then transformed into *E.coli* NM522. The samples were plated and individual colonies were analyzed for inserts of appropriate size. Because a 1 kb insert

n gentle rocking in Alkaline Transfer Solution for 30 min. Dye
olour.

er Solution: NaOH 0.4 M
NaCl 0.6 M

N+ membrane (DuPont Co.) on Alkaline Transfer Solution for 10
tare.

t in pyrex tray as: 7 sheets of blotting paper
gel
Hybond N+ membane
3 sheets of filter paper
2 cm thick layer of paper towels
200 g weight

Transfer Solution to the pyrex tray and replenish when required for
t room temperature.

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s et al., 1982)

ne between layers of blotting paper and bake at 85C for 2 h.

A FOR PROBES

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s or from the cDNA library produced by Clontech Laboratories Inc.
t PBI from Laird lentils. In this library, cDNA fragments were cloned
of a λgt11 cloning vector as was described in Report #2. The library
individual plaques stored in 1 ml of SM medium with a drop of
1982) for future use. The protocol for isolating λDNA from these
as follows:

4 plates, add 100 µl of an overnight culture of Y1090 plating
a single plaque λgt11 isolate to 4 ml of cooled 0.7% agarose, Mix
over plates of NZCYM medium containing 30 µg ml^{-1}.

37C overnight.

the top agar of plates with clearly visible plaques into a 50 ml centrifuge
l of λ dilution buffer (10 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM

0 rpm) at room temperature for 2 h on rotary shaker.

fuge at 16,000 x g for 30 min at 4C.

pernatant into clean tube and repeat the centrifugation.

d 20 µl of RNAase
nin.

8000, 2 M NaCl in

1 500 µl of TEN

:entrifuge 5 min.

vortex for 5

stand at -20C for

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with a high salt
7T318U
plated and
1 kb insert

would represent about 25% of the DNA, sufficient insert DNA for several probes could be recovered from gels when the DNA from 2 minipreps was digested with EcoRI.

PREPARATION OF RADIOACTIVE PROBES

^{32}P labeled probes for detection of RFLP hybridization bands were prepared using the random primers method as provided in kit form by Boeheringer Mannheim. For a single gel described above (2 membranes) the following protocol and reagents were used.

1. Combine the probe DNA ($\approx$100 ng), pure λ DNA (100 pg) and water to a volume of 18 μl. Boil 10 min and cool on ice.

2. Add 6 μl of dATP, dGTP, dTTP mixture, 4 μl of reaction buffer x 10, 10 μl of dCTP (100 μCi) and 2 μl of Klenow fragment. Mix and incubate for 1.5 h at 37C.

3. Remove unincorporated dCTP using either Elutip Columns (Schleicher & Schuell) according to the manufacturer's instructions or Sephadex G50 spin columns as described in Maniatis et al (1982).

HYBRIDIZATION TO MEMBRANE BOUND DNA

Southern hybridizations were incubated in plastic boxes in a water bath or in 26 cm x 3.5 cm i.d. sealed tubes in a hybridization incubator (Robbins Scientific). Except for the volumes of solution used, the methods were the same.

The two membranes containing all samples from a single large gel were placed into either a single incubation box (membranes placed with DNA sides away from each other) or into two incubation tubes. For each box, 80 ml of the following prehybridization solution were added and for tube, 10 ml were used.

Hybridization buffer (50 mM Tris/HCl, pH 7.5, 1M NaCl)	16 ml
Deionized formamide	40 ml
50% Dextran Sulfate	16 ml
10% Sodium dodecyl sulfate	8 ml

Prehybridization was carried out for approximately 5 h at 42C with gentle agitation of the boxes or rotation of the glass hybridization tubes.

Probes to be used were heated to 100C for 10 min and then cooled on ice before being added to the reaction boxes or tubes. Hybridization was for 20 h at 42C.

The hybridization solution was removed from the hybridization boxes and tubes and the membranes were treated with the following wash treatments with vigorous agitation.

2 x 100 ml of SSC (Maniatis et al., 1982) at room temperature for 5 min.
2 x 200 ml of SSC + 1% SDS at 65C for 30 min.
2 x 100 ml 0.1 SSC at room temperature for 30 min.

Membranes were blotted briefly and sealed into plastic bags. The bags were placed on X-ray film in light-tight cassettes and exposed for from 5 to 15 days, after which the films were developed.

APPENDIX 4.

PCR AMPLIFICATION AND SEPARATION OF RAPD MARKERS

Appendix 4. PCR AMPLIFICATION AND SEPARATION OF RAPD MARKERS

For a polymerase chain reaction to work with a single primer, there must be two primer binding sites on opposite strands of the DNA within the 5 kb effective size limitation of the reaction. The appearance of amplified bands can also be affected by the reaction conditions (including those introduced as impurities in DNA samples). However, for a large scale screening of primers, uniform conditions were used and the following conditions were defined by Dr. L. Pelcher of the DNA Services Unit after preliminary tests of several primers with lentil DNA.

Reaction Mix:	PCR Buffer x 10	100 mM KCl 100 mM $(NH_4)_2SO_4$ 200 mM Tris/HCl pH 8.8 20 mM $MgSO_4$ 1% Triton X-100)	5.0 µl
	water		32.5 µl
	dNTP mix (1.25 mM)		8.0 µl
	primer 10-mer 15.2 pmol μl^{-1}		2.5 µl
	9-mer 17.0 pmol μl^{-1}		
	plant DNA (200 ng μl^{-1})		1.0 µl
	Taq polymerase (1.25 u μl^{-1}		1.0 µl

Reaction mixtures were covered with mineral oil and the thermal cycling was begun as outlined below using a Perkin Elmer DNA Thermal Cycler.

Programmed Reaction Conditions	35 Cycles	96C	2 min
		94C	30 sec
		37C	1 min
		73C	2 min
	1 Cycle	73C	7 min
		15C	Hold

After the PCR reactions were completed, the reaction mixtures were removed from below the mineral oil (usually 45 µl) and mixed with 12 µl of loading buffer containing EDTA, methylene blue dye and glycerol. The PCR reaction products were separated on agarose gels (1.2% with ethidium bromide in TBE buffer). The 400 mL gels were cast in 20 x 24 cm bridges with 2 x 30 well combs, so that 60 samples could be run at one time. For the initial screening of RAPD primers, 20 samples were run on each half of the gel along with two samples of a 1kb standard size ladder for size comparison. For analyzing the parent and progeny populations, DNA from 2 plants of each parent type and 20 F_2 plants were run with two 1 kb ladder standards on each half of the gel. When sufficient separation of the bands had been achieved (as visualized by ethidium bromide fluorescence on a uv light box), the banding patterns were photographed with a Polaroid system and Type 55 film so that positive prints were available for identification of bands and negatives were available for densitometer analysis or for later preparation of positive images.

APPENDIX 5.

ASCOCHYTA INFECTION IN RELATION TO RFLP AND RAPD MARKERS

These tables present the data for comparison of *Ascochyta* resistance with the RFLP and RAPD molecular markers.

ASCOCHYTA AND MARKERS

This presents all data collected on *Ascochyta* infection and the scoring of all RFLP and RAPD markers from the 4 plants of each parent and 40 F_2 progeny. RFLP markers scored as 1 for presence of Eston band, 0 for presence of ILL5588 band and 0.5 for both. RAPD markers scored as 1 for presence of polymorphic band and 0 for absence. For each marker, the numbers of genotypes in each class and the χ^2 value and probability are presented from comparisons to a 3:1ratio for RAPD markers or 1:2:1 ratio for RFLP markers.

CORRELATION GRID

Ascochyta infection and marker data for parent plants and the 28 F_2 plants for which complete data are available. RFLP results scored as 1 if similar to Eston, 0 if similar to ILL5588 and 0.5 if both bands present. RAPD results scored as 1 if similar to Eston and 0 if similar to ILL5588. Also presented are numbers in each class for each marker and correlation coefficients between each set of marker scores and the linear or ln transformed *Ascochyta* results.

CORRELATION COEFFICIENTS

Correlation coefficients for comparisons of F_2 scores for *Ascochyta* infection and RFLP or RAPD markers based on the data in the CORRELATION GRID. All pairs of scores were compared and *, ** mean significance at $P<0.05$ and $P<0.01$ respectively. SASLIN28 - linear values for Ascochyta infection, SASLN28 - ln transformed values for Ascochyta infection, RFLP markers specified by restriction enzyme (BCL= BclI, BGL=BglII) and the probe (528=LRF5, 1528=LRF15, 928=LRF9), and RAPD markers are specified by the primer number and the polymorphism number (e.g. SU516228 =polymorphic band 2 from primer U516).

RFLP AND RAPD COMBINATIONS

Data from 33 F_2 progeny plants on *Ascochyta* infection and scores for 2 RFLP and 4 RAPD markers. Marker scores are based on estimates of resistance-gene copy numbers if markers were linked to resistance as described inthe text. Combination scores were simple sums of the scores from 2 or 3 markers and all data were divided into three groups corresponding to high, medium and low *Ascochyta* infection.

SAMPLE				SEED INFECTION		RFLP POLYMORPHISMS			
						ENZYME	BclI	BglII	BglII	BclI
						Probe	LRF5	LRF5	LRF15	LRF9
						Size	2.3kb=1	7.4kb=1	6.7kb=1	5.6kb=1
				Mean	Mean		1.6kb=0	9.9kb=0	12.7kb=0	14.2kb=0
Anal#	BI	Gen	PI	lin	ln					
E1	1	1	3	58.8	4.05		1	1	1	1
E2	1	1	12	51.7	3.81		1	1	1	1
E3	1	1	13	59.2	3.52		1	1		1
E4	1	1	14	46.9	3.74		1	1	1	1
I1	1	3	4	1.3	0.30		0	0	0	0
I2	1	3	10	2.0	0.30		0	0	0	0
I3	2	3	4	0.0	0.00		0	0	0	0
I4	2	3	7	0.8	0.20		0	0	0	0
F10	1	4	13	96.0	4.57		0.5	0.5	0.5	0.5
F7	1	4	10	83.0	4.40		0.5	0.5	0.5	1
F24	2	4	18	52.5	3.64		0	0	0	0
F8	1	4	11	50.9	3.73		1	1	0	0.5
F23	2	4	17	46.7	3.50		0.5	0.5	0	0.5
F2	1	4	3	33.3	2.62		1	1	1	0
F4	1	4	6	14.3	2.42		0.5	0.5	0.5	0.5
F18	2	4	7	12.9	1.68		0	0	0.5	0.5
F5	1	4	8	11.0	1.70		1	1	0.5	0.5
F26	2	4	22	10.0	1.86		1	1	0	1
F39	2	5	22	8.3	1.68		0.5	0.5	0.5	1
F20	2	4	14	5.8	0.79		1	1	0.5	0.5
F9	1	4	12	5.0	0.53		1	1	0.5	0.5
F35	2	5	7	5.0	0.53		0	0	0.5	0.5
F15	2	4	2	4.0	0.78		0.5	0.5	0	0.5
F29	1	5	7	3.8	0.90		0.5	0.5	1	0.5
F30	1	5	8	3.3	0.80		0	0	1	0
F37	2	5	13	3.3	0.80		0.5	0.5	0.5	0
F6	1	4	9	3.0	0.72		0	0	1	1
F17	2	4	6	2.5	0.45		0.5	0.5	0.5	1

Anal#	Bl	Gen	Pl	Mean lin	Mean ln		Bcl I LRF5	Bgl II LRF5	Bgl II LRF15	BclI LRF9
F1	1	4	2	1.7	0.40		0	0	1	0.5
F19	2	4	10	1.7	0.40		0.5	0.5	0.5	0.5
F22	2	4	16	1.7	0.40		0	0	0	0.5
F25	2	4	20	1.7	0.40		0	0	1	0
F34	2	5	2	1.7	0.40		0.5	0.5	1	0.5
F12	1	4	15	1.3	0.30		1	1	X	0.5
F33	1	5	13	1.3	0.30		0.5	0.5	0	0.5
F3	1	4	5	0.8	0.20		0.5	0.5	0.5	0
F11	1	4	14	0.8	0.20		0	0	0	0
F32	1	5	11	0.8	0.20		0.5	0.5	0.5	1
F36	2	5	12	0.8	0.20		1	1	0.5	0.5
F13	1	4	17	0.0	0.00		0	0	0.5	0
F14	2	4	1	0.0	0.00		0.5	0.5	0.5	0
F16	2	4	4	0.0	0.00		0.5	0.5	0.5	X
F21	2	4	15	0.0	0.00		0	0	0.5	1
F27	2	4	23	0.0	0.00		0.5	0.5	0.5	1
F28	1	5	4	0.0	0.00		0.5	0.5	1	0
F31	1	5	10	0.0	0.00		0.5	0.5	0.5	1
F38	2	5	20	0.0	0.00		0.5	0.5	0	0
F40	2	5	23	0.0	0.00		0	0	0.5	0
						#=0	12	12	9	12
						#=0.5	20	20	22	18
						#=1	8	8	8	9
						X2	0.80	0.80	0.69	0.48
						P	<.67	<.67	<.71	<.78

	RAPD POLYMORPHISMS								
Primer	rc41	516	516.2	516.3	519.1	519.2	521	535	540.1
Size	680	1220	1490	760	1990	660	540	1200	500
	1	0	1	1	1	1	1	0	1
	1	0	1	1	1	1	1	0	1
	1	0	1	1	1	1	1	0	1
	1	0	1	1	1	1	1	0	1
	0	1	0	0	0	0	0	1	0
	0	1	0	0	0	0	0	1	0
	0	1	0	0	0	0	0	1	0
	0	1	0	0	X	X	X	1	X
	1	1	0	0	0	1	1	1	1
	X	1	1	1	1	0	1	1	1
	1	0	0	1	0	1	1	1	0
	1	1	0	1	1	1	0	1	1
	1	1	1	1	1	1	0	0	1
	1	0	0	0	0	0	1	1	1
	0	0	1	0	1	1	1	0	1
	1	1	1	1	1	1	1	1	1
	1	1	0	1	0	1	1	0	1
	0	0	1	0	1	1	1	0	1
	1	1	1	1	1	0	1	1	1
	1	1	1	1	1	1	1	0	0
	1	1	1	1	1	1	1	0	0
	1	1	1	1	1	1	1	1	0
	X	1	0	1	0	1	0	1	1
	1	0	1	1	1	1	1	1	0
	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	0	0	1	0
	0	0	0	0	0	1	1	1	0
	1	0	1	1	1	1	1	0	X

	rc41	516	516.2	516.3	519.1	519.2	521	535	540.1
	680	1220	1490	760	1990	660	540	1200	500
	0	0	1	1	1	1	1	1	0
	1	1	1	1	1	1	1	1	1
	1	1	0	1	0	1	0	1	1
	0	0	1	1	1	1	1	1	0
	0	1	1	1	1	1	1	1	1
	1	1	1	1	1	0	1	1	1
	0	1	1	1	1	1	0	1	1
	1	1	0	1	0	1	1	0	0
	1	1	1	1	1	0	0	1	1
	1	1	1	1	1	1	1	1	0
	1	X	X	X	1	1	1	X	X
	1	1	1	1	1	1	0	1	1
	0	0	1	0	1	1	1	1	1
	0	1	0	1	0	1	1	1	1
	1	1	1	1	1	0	0	1	1
	0	1	1	1	X	X	X	X	X
	1	1	0	1	0	1	1	1	0
	0	1	0	0	1	1	1	1	1
	1	X	X	X	1	1	0	X	0
	1	X	X	X	X	X	X	X	X
#=0	11	10	12	7	10	7	10	8	13
#=1	27	27	25	30	28	31	28	29	23
	0.50	0.08	1.09	0.73	0.03	0.88	0.88	0.22	2.37
	<.48	<.78	<0.30	<.39	<.85	<.34	<.35	<.66	<.12

RAPD POLYMORPHISMS							
540.2	540.3	562	573	584	585	598	599
1070	1280	1200	320	680	730	1000	1130
0	0	0	1	1	1	X	1
0	0	0	1	1	1	1	1
0	0	0	1	1	1	1	1
0	0	0	1	1	1	1	1
1	1	1	0	0	0	0	0
1	1	1	0	0	X	0	0
1	1	1	0	0	0	0	0
X	X	1	0	0	X	X	X
1	1	X	1	0	0	0	0
1	1	1	1	1	1	1	0
1	1	1	1	0	0	0	1
1	1	1	0	1	1	1	0
1	1	1	0	1	0	1	0
1	1	X	1	1	0	0	1
1	0	0	1	1	0	1	1
1	1	1	1	1	1	0	1
1	0	1	1	1	0	0	1
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
1	0	X	1	1	X	1	1
0	1	1	1	1	1	1	1
0	0	X	1	0	1	0	1
1	1	1	0	1	1	1	0
0	0	0	1	0	0	1	1
1	1	0	1	1	0	1	0
0	1	1	0	0	1	0	0
0	1	X	1	0	1	0	1
X	X	X	1	X	X	X	1

540.2	540.3	562	573	584	585	598	599
1070	1280	1200	320	680	730	1000	1130
0	0	0	1	1	1	1	1
1	1	1	1	1	1	1	1
1	1	1	0	1	1	0	0
0	1	X	1	1	1	0	X
0	0	1	1	1	1	1	1
0	1	1	1	1	1	0	0
1	1	1	0	0	1	0	1
1	1	1	1	0	0	0	1
1	1	1	0	1	1	0	0
1	1	1	1	1	1	1	1
X	X	X	X	X	X	0	X
0	1	1	0	1	1	0	1
1	1	1	1	1	1	1	0
0	0	0	0	1	1	1	0
1	1	X	0	1	1	1	0
X	X	1	X	X	X	X	X
0	1	X	X	0	0	1	1
1	1	1	1	1	0	1	1
0	1	X	1	X	X	0	1
X	X	X	X	X	X	X	X
13	7	5	10	9	11	17	13
23	27	23	26	26	23	20	23
2.37	0.79	0.76	0.15	0.09	0.98	8.66	2.37
<.12	<.38	<.38	<.70	<.93	<.32	<.01*	<.12

						ENZYME	Bcl I	Bgl II	Bgl II	Bcl I
						Probe	LRF5	LRF5	LRF15	LRF9
						Size	2.3, 1.6 Kb	7.4, 9.9	6.7, 12.6	5.6, 14.2
				Mean	Mean					
Anal#	Bl	Gen	Pl	lin	ln					
E1	1	1	3	58.8	4.05		2.3	7.4	6.7	5.6
E2	1	1	12	51.7	3.81		2.3	7.4	6.7	5.6
E3	1	1	13	59.2	3.52		2.3	7.4	6.7	5.6
E4	1	1	14	46.9	3.74		2.3	7.4	6.7	5.6
I1	1	3	4	1.3	0.30		1.6	9.9	12.7	14.2
I2	1	3	10	2.0	0.30		1.6	9.9	12.7	14.2
I3	2	3	4	0.0	0.00		1.6	9.9	12.7	14.2
I4	2	3	7	0.8	0.20		1.6	9.9	12.7	14.2
F10	1	4	13	96.0	4.57		0.5	0.5	0.5	0.5
F8	1	4	11	50.9	3.73		1	1	0	0.5
F24	2	4	18	52.5	3.64		0	0	0	0
F23	2	4	17	46.7	3.50		0.5	0.5	0	0.5
F2	1	4	3	33.3	2.62		1	1	1	0
F4	1	4	6	14.3	2.42		0.5	0.5	0.5	0.5
F26	2	4	22	10.0	1.86		1	1	0	1
F5	1	4	8	11.0	1.70		1	1	0.5	0.5
F18	2	4	7	12.9	1.68		0	0	0.5	0.5
F39	2	5	22	8.3	1.68		0.5	0.5	0.5	1
F9	1	4	12	5.0	0.53		1	1	0.5	0.5
F35	2	5	7	5.0	0.53		0	0	0.5	0.5
F29	1	5	7	3.8	0.90		0.5	0.5	1	0.5
F30	1	5	8	3.3	0.80		0	0	1	0
F37	2	5	13	3.3	0.80		0.5	0.5	0.5	0
F6	1	4	9	3.0	0.72		0	0	1	1
F1	1	4	2	1.7	0.40		0	0	1	0.5
F19	2	4	10	1.7	0.40		0.5	0.5	0.5	0.5
F22	2	4	16	1.7	0.40		0	0	0	0.5
F34	2	5	2	1.7	0.40		0.5	0.5	1	0.5
F33	1	5	13	1.3	0.30		0.5	0.5	0	0.5
F3	1	4	5	0.8	0.20		0.5	0.5	0	0
F11	1	4	14	0.8	0.20		0	0	0	0
F32	1	5	11	0.8	0.20		0.5	0.5	0.5	1
F13	1	4	17	0.0	0.00		0	0	0.5	0
F14	2	4	1	0.0	0.00		0.5	0.5	0.5	0
F21	2	4	15	0.0	0.00		0	0	0.5	1
F31	1	5	10	0.0	0.00		0.5	0.5	0.5	1
							1 = similar to Eston		0 = similar to ILL5588	
			0.323 - P<0.10			#=1	5	5	6	6
			0.374 - P<0.05			#=0.5	13	13	14	14
			0.478 - P<0.01			#=0	10	10	8	8
			Correlation			r lin	0.22	0.22	-0.21	-0.10
			Coefficients			r ln	0.3	0.30	-0.21	-0.06

APPENDIX 5. CORRELATION GRID 28 PLANTS

Primer	rc41	516	516.2	516.3	519.1	519.2	521	535
Size	680	1220	1490	760	1990	660	540	1200
		P= presence						
		A=absence						
E1	P	A	P	P	P	P	P	A
E2	P	A	P	P	P	P	P	A
E3	P	A	P	P	P	P	P	A
E4	P	A	P	P	P	P	P	A
I1	A	P	A	A	A	A	A	P
I2	A	P	A	A	A	A	A	P
I3	A	P	A	A	A	A	A	P
I4	A	P	A	A	X	X	X	P
F10	1	0	0	0	0	1	1	0
F8	1	0	0	1	1	1	0	0
F24	1	1	0	1	0	1	1	0
F23	1	0	1	1	1	1	0	1
F2	1	1	0	0	0	0	1	0
F4	0	1	1	0	1	1	1	1
F26	0	1	1	0	1	1	1	1
F5	1	0	0	1	0	1	1	1
F18	1	0	1	1	1	1	1	0
F39	1	0	1	1	1	0	1	0
F9	1	0	1	1	1	1	1	1
F35	1	0	1	1	1	1	1	0
F29	1	1	1	1	1	1	1	0
F30	1	0	1	1	1	1	1	0
F37	1	0	1	1	1	0	0	0
F6	0	1	0	0	0	1	1	0
F1	0	1	1	1	1	1	1	0
F19	1	0	1	1	1	1	1	0
F22	1	0	0	1	0	1	0	0
F34	0	0	1	1	1	1	1	0
F33	0	0	1	1	1	1	0	0
F3	1	0	0	1	0	1	1	1
F11	1	0	1	1	1	0	0	0
F32	1	0	1	1	1	1	1	0
F13	1	0	1	1	1	1	0	0
F14	0	1	1	0	1	1	1	0
F21	1	0	1	1	1	0	0	0
F31	0	0	0	0	1	1	1	0
#=0	8	20	9	7	7	5	8	22
#=1	20	8	19	21	21	23	20	6
r lin	0.26	0.05	-0.45	-0.24	-0.39	0.09	0.00	0.03
r ln	0.23	0.16	-0.39	-0.22	-0.34	0.06	0.06	0.18

APPENDIX 5: CORRELATION GRID 28 PLANTS

	540.1	540.2	540.3	573	584	585	598	599
	500	1070	1280	320	680	730	1000	1130
E1	P	A	A	P	P	P	X	P
E2	P	A	A	P	P	P	P	P
E3	P	A	A	P	P	P	P	P
E4	P	A	A	P	P	P	P	P
I1	A	P	P	A	A	A	A	A
I2	A	P	P	A	A	X	A	A
I3	A	P	P	A	A	A	A	A
I4	X	X	X	A	A	X	X	X
F10	1	0	0	1	0	0	0	0
F8	1	0	0	0	1	1	1	0
F24	0	0	0	1	0	0	0	1
F23	1	0	0	0	1	0	1	0
F2	1	0	0	1	1	0	0	1
F4	1	0	1	1	1	0	1	1
F26	1	0	0	1	1	1	1	1
F5	1	0	1	1	1	0	0	1
F18	1	0	0	1	1	1	0	1
F39	1	0	0	1	1	1	1	1
F9	0	1	0	1	1	1	1	1
F35	0	1	1	1	0	1	0	1
F29	0	1	0	1	0	0	1	1
F30	1	0	1	1	1	0	1	0
F37	0	1	0	0	0	1	0	0
F6	0	1	0	1	0	1	0	1
F1	0	1	1	1	1	1	1	1
F19	1	0	0	1	1	1	1	1
F22	1	0	0	0	1	1	0	0
F34	1	1	1	1	1	1	1	1
F33	1	0	0	0	0	1	0	1
F3	0	0	0	1	0	0	0	1
F11	1	0	0	0	1	1	0	0
F32	0	0	0	1	1	1	1	1
F13	1	1	0	0	1	1	0	1
F14	1	0	0	1	1	1	1	0
F21	1	0	0	0	1	1	1	0
F31	1	0	0	1	1	0	1	1
	9	20	22	8	8	10	13	9
	19	8	6	20	20	18	15	19
	0.15	-0.29	-0.17	0.00	-0.21	-0.44	-0.16	-0.29
	0.18	-0.33	-0.07	0.06	-0.12	-0.47	-0.10	-0.17

APPENDIX 5. CORRELATION COEFFICIENTS

ASCOCHYTA INFECTION VS RFLP AND RAPD MARKERS 28 F2 PLANTS

05-21-1992 COMMON PATH FOR FILES IS C:\EXCEL\ADFDAT\

TABLE CALCULATED WITH 26 DEGREES OF FREEDOM

	:SASLIN28	:SASLN28	:SBCL528	:SBGL528	:SBGL1528	:SBCL928
	:SRC4128	:SU51628	:SU516228	:SU516328	:SU519128	:SU519228
	:SU52128	:SU53528	:SU540128	:SU540228	:SU540328	:SU57328
	:SU58428	:SU58528	:SU59828	:SU59928		
SASLIN28	: 1.000	:+0.926**	:+0.217	:+0.217	:-0.210	:-0.104
	:+0.263	:+0.046	:-0.450*	:-0.239	:-0.394*	:+0.086
	:+0.003	:+0.034	:+0.148	:-0.293	:-0.166	:+0.003
	:-0.214	:-0.436*	:-0.161	:-0.289		
SASLN28	: -	: 1.000	:+0.295	:+0.295	:-0.210	:-0.064
	:+0.234	:+0.164	:-0.388*	:-0.220	:-0.342	:+0.058
	:+0.056	:+0.181	:+0.175	:-0.334	:-0.072	:+0.056
	:-0.121	:-0.472*	:-0.097	:-0.172		
SBCL528	: -	: -	: 1.000	:+1.000**	:-0.097	:+0.118
	:-0.048	:+0.048	:-0.173	:-0.261	:-0.029	:+0.014
	:+0.175	:+0.499**	:+0.150	:-0.175	:-0.114	:+0.175
	:+0.175	:-0.188	:+0.270	:+0.150		
SBGL528	: -	: -	: -	: 1.000	:-0.097	:+0.118
	:-0.048	:+0.048	:-0.173	:-0.261	:-0.029	:+0.014
	:+0.175	:+0.499**	:+0.150	:-0.175	:-0.114	:+0.175
	:+0.175	:-0.188	:+0.270	:+0.150		
SBGL1528	: -	: -	: -	: -	: 1.000	:+0.062
	:-0.177	:+0.289	:+0.148	:-0.176	:+0.059	:-0.047
	:+0.498**	:-0.318	:-0.179	:+0.514**	:+0.424*	:+0.498**
	:+0.048	:-0.076	:+0.211	:+0.256		
SBCL928	: -	: -	: -	: -	: -	: 1.000
	:-0.289	:-0.048	:+0.039	:-0.176	:+0.176	:+0.085
	:+0.161	:+0.053	:+0.039	:-0.048	:-0.071	:+0.161
	:+0.161	:+0.242	:+0.414*	:+0.256		

SRC4128	–	–	–	–	–	–
	–	–	–	–	–	–
	1.000	-0.475*	-0.097	+0.548**	-0.183	-0.295
	-0.225	-0.055	-0.097	-0.125	-0.248	-0.225
	-0.050	-0.141	-0.272	-0.266		
SU51628	–	–	–	–	–	–
	–	1.000	-0.073	-0.548**	-0.183	+0.088
	+0.4*	+0.055	-0.242	+0.125	+0.055	+0.4*
	-0.125	-0.189	+0.113	+0.266		
SU516228	–	–	–	–	–	–
	–	–	1.000	+0.309	+0.839**	-0.121
	-0.097	-0.013	+0.018	+0.266	+0.173	-0.097
	+0.242	+0.445*	+0.433*	+0.018		
SU516328	–	–	–	–	–	–
	–	–	–	1.000	+0.238	-0.054
	-0.365	-0.101	-0.221	+0.183	+0.101	-0.365
	+00	+0.258	-0.041	-0.044		
SU519128	–	–	–	–	–	–
	–	–	–	–	1.000	-0.054
	-0.183	-0.101	+0.132	+0.183	+0.101	-0.183
	+0.365	+0.430*	+0.620**	-0.044		
SU519228	–	–	–	–	–	–
	–	–	–	–	–	1.000
	+0.324	+0.243	-0.121	+0.088	+0.243	+0.324
	-0.088	-0.153	+0.127	+0.278		
SU52128	–	–	–	–	–	–
	–	–	–	–	–	–
	1.000	+0.138	-0.266	+0.050	+0.330	+1.000**
	-0.050	-0.306	+0.204	+0.580**		
SU53528	–	–	–	–	–	–
	–	–	–	–	–	–
	–	1.000	-0.013	-0.138	+0.152	+0.138
	+0.138	-0.337	+0.137	+0.173		

SU540128	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	1.000	-0.580**	-0.013	-0.266	
	+0.580**	-0.034	+0.126	-0.310				
SU540228	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	1.000	+0.248	+0.050	
	-0.3	+0.306	-0.045	+0.266				
SU540328	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	1.000	+0.330	
	+0.138	-0.156	+0.137	+0.173				
SU57328	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	1.000	
	-0.050	-0.306	+0.204	+0.580**				
SU58428	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	1.000	+0.189	+0.521**	-0.097				
SU58528	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	1.000	+0.053	-0.034				
SU59828	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	1.000	-0.027				
SU59928	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
	-	-	-	-	1.000			

PLANT	ASCO lin	ASCO ln	BclI LRF5	BglII LRF15	RC41	516	521
10	96.0	4.57	1	1	0.5	1.5	0.5
24	52.5	3.64	2	2	0.5	0	0.5
8	50.9	3.73	0	2	0.5	1.5	2
23	46.7	3.5	1	2	0.5	1.5	2
2	33.3	2.62	0	0	0.5	0	0.5
4	14.3	2.42	1	1	2	0	0.5
18	12.9	1.68	2	1	0.5	1.5	0.5
5	11.0	1.7	0	1	0.5	1.5	0.5
26	10.0	1.86	0	2	2	0	0.5
39	8.3	1.68	1	1	0.5	1.5	0.5
20	5.8	0.79	0	1	0.5	1.5	0.5
	31.06	2.56	0.73	1.27	0.77	0.95	0.77
9	5.0	0.53	0	1	0.5	1.5	0.5
35	5.0	0.53	2	1	0.5	1.5	0.5
29	3.8	0.9	1	0	0.5	0	0.5
30	3.3	0.8	2	0	0.5	1.5	0.5
37	3.3	0.8	1	1	0.5	1.5	2
6	3.0	0.72	2	0	2	0	0.5
17	2.5	0.45	1	1	0.5	0	0.5
1	1.7	0.4	2	0	0.5	0	0.5
19	1.7	0.4	1	1	0.5	1.5	0.5
22	1.7	0.4	2	2	0.5	1.5	2
25	1.7	0.4	2	0	2	0	0.5
	2.97	0.58	1.45	0.64	0.77	0.82	0.77
34	1.7	0.4	1	0	2	1.5	0.5
33	1.3	0.3	1	2	2	1.5	2
3	0.8	0.2	1	1	0.5	1.5	0.5
11	0.8	0.2	2	1	0.5	1.5	2
32	0.8	0.2	1	1	0.5	1.5	0.5
13	0.0	0	2	1	0.5	1.5	2
14	0.0	0	1	1	2	0	0.5
16	0.0	0	1	1	0.5	1.5	2
21	0.0	0	2	0	0.5	1.5	2
28	0.0	0	1	1	0.5	1.5	0.5
31	0.0	0	1	1	2	1.5	0.5
	0.49	0.12	1.27	0.91	1.05	1.36	1.18

In order to give some indication of resistance dosage, the data were entered as follows

For RFLP Markers in the F2 0=2, .5=1, 1=0

For RAPD Markers If ILL5588=1 1 in F2 =1.5

0 in F2 = 0

If ILL5588=0 0 in F2 = 2

1 in F2=.5

APPENDIX 5. RFLP AND RAPD COMBINATIONS

PLANT	535	LRF5 LRF15	LRF5 RC41	LRF5 516	LRF5 521	LRF5 535	LRF15 RC41
10	1.5	2	1.5	2.5	1.5	2.5	1.5
24	1.5	4	2.5	2	2.5	3.5	2.5
8	1.5	2	0.5	1.5	2	1.5	2.5
23	0	3	1.5	2.5	3	1	2.5
2	1.5	0	0.5	0	0.5	1.5	0.5
4	0	2	3	1	1.5	1	3
18	1.5	3	2.5	3.5	2.5	3.5	1.5
5	0	1	0.5	1.5	0.5	0	1.5
26	0	2	2	0	0.5	0	4
39	1.5	2	1.5	2.5	1.5	2.5	1.5
20	0	1	0.5	1.5	0.5	0	1.5
	0.82	2.00	1.50	1.68	1.50	1.55	2.05
9	0	1	0.5	1.5	0.5	0	1.5
35	1.5	3	2.5	3.5	2.5	3.5	1.5
29	1.5	1	1.5	1	1.5	2.5	0.5
30	1.5	2	2.5	3.5	2.5	3.5	0.5
37	1.5	2	1.5	2.5	3	2.5	1.5
6	1.5	2	4	2	2.5	3.5	2
17	0	2	1.5	1	1.5	1	1.5
1	1.5	2	2.5	2	2.5	3.5	0.5
19	1.5	2	1.5	2.5	1.5	2.5	1.5
22	1.5	4	2.5	3.5	4	3.5	2.5
25	1.5	2	4	2	2.5	3.5	2
	1.23	2.09	2.23	2.27	2.23	2.68	1.41
34	1.5	1	3	2.5	1.5	2.5	2
33	1.5	3	3	2.5	3	2.5	4
3	0	2	1.5	2.5	1.5	1	1.5
11	1.5	3	2.5	3.5	4	3.5	1.5
32	1.5	2	1.5	2.5	1.5	2.5	1.5
13	1.5	3	2.5	3.5	4	3.5	1.5
14	1.5	2	3	1	1.5	2.5	3
16	1.5	2	1.5	2.5	3	2.5	1.5
21	1.5	2	2.5	3.5	4	3.5	0.5
28	1.5	2	1.5	2.5	1.5	2.5	1.5
31	1.5	2	3	2.5	1.5	2.5	3
	1.36	2.18	2.32	2.64	2.45	2.64	1.95

PLANT	LRF15 516	LRF15 521	LRF15 535	RC41 516	RC41 521	RC41 535	516 521
10	2.5	1.5	2.5	2	1	2	2
24	2	2.5	3.5	0.5	1	2	0.5
8	3.5	4	3.5	2	2.5	2	3.5
23	3.5	4	2	2	2.5	0.5	3.5
2	0	0.5	1.5	0.5	1	2	0.5
4	1	1.5	1	2	2.5	2	0.5
18	2.5	1.5	2.5	2	1	2	2
5	2.5	1.5	1	2	1	0.5	2
26	2	2.5	2	2	2.5	2	0.5
39	2.5	1.5	2.5	2	1	2	2
20	2.5	1.5	1	2	1	0.5	2
	2.23	2.05	2.09	1.73	1.55	1.59	1.73
9	2.5	1.5	1	2	1	0.5	2
35	2.5	1.5	2.5	2	1	2	2
29	0	0.5	1.5	0.5	1	2	0.5
30	1.5	0.5	1.5	2	1	2	2
37	2.5	3	2.5	2	2.5	2	3.5
6	0	0.5	1.5	2	2.5	3.5	0.5
17	1	1.5	1	0.5	1	0.5	0.5
1	0	0.5	1.5	0.5	1	2	0.5
19	2.5	1.5	2.5	2	1	2	2
22	3.5	4	3.5	2	2.5	2	3.5
25	0	0.5	1.5	2	2.5	3.5	0.5
	1.45	1.41	1.86	1.59	1.55	2.00	1.59
34	1.5	0.5	1.5	3.5	2.5	3.5	2
33	3.5	4	3.5	3.5	4	3.5	3.5
3	2.5	1.5	1	2	1	0.5	2
11	2.5	3	2.5	2	2.5	2	3.5
32	2.5	1.5	2.5	2	1	2	2
13	2.5	3	2.5	2	2.5	2	3.5
14	1	1.5	2.5	2	2.5	3.5	0.5
16	2.5	3	2.5	2	2.5	2	3.5
21	1.5	2	1.5	2	2.5	2	3.5
28	2.5	1.5	2.5	2	1	2	2
31	2.5	1.5	2.5	3.5	2.5	3.5	2
	2.27	2.09	2.27	2.41	2.23	2.41	2.55

APPENDIX 5. RFLP AND RAPD COMBINATIONS

PLANT	516 535	521 535	LRF5 RC41 516	LRF5 RC41 521	LRF5 RC41 535	LRF5 516 521	LRF5 516 535
10	3	2	3	2	3	3	4
24	1.5	2	2.5	3	4	2.5	3.5
8	3	3.5	2	2.5	2	3.5	3
23	1.5	2	3	3.5	1.5	4.5	2.5
2	1.5	2	0.5	1	2	0.5	1.5
4	0	0.5	3	3.5	3	1.5	1
18	3	2	4	3	4	4	5
5	1.5	0.5	2	1	0.5	2	1.5
26	0	0.5	2	2.5	2	0.5	0
39	3	2	3	2	3	3	4
20	1.5	0.5	2	1	0.5	2	1.5
	1.77	1.59	2.45	2.27	2.32	2.45	2.50
9	1.5	0.5	2	1	0.5	2	1.5
35	3	2	4	3	4	4	5
29	1.5	2	1.5	2	3	1.5	2.5
30	3	2	4	3	4	4	5
37	3	3.5	3	3.5	3	4.5	4
6	1.5	2	4	4.5	5.5	2.5	3.5
17	0	0.5	1.5	2	1.5	1.5	1
1	1.5	2	2.5	3	4	2.5	3.5
19	3	2	3	2	3	3	4
22	3	3.5	4	4.5	4	5.5	5
25	1.5	2	4	4.5	5.5	2.5	3.5
	2.05	2.00	3.05	3.00	3.45	3.05	3.50
34	3	2	4.5	3.5	4.5	3	4
33	3	3.5	4.5	5	4.5	4.5	4
3	1.5	0.5	3	2	1.5	3	2.5
11	3	3.5	4	4.5	4	5.5	5
32	3	2	3	2	3	3	4
13	3	3.5	4	4.5	4	5.5	5
14	1.5	2	3	3.5	4.5	1.5	2.5
16	3	3.5	3	3.5	3	4.5	4
21	3	3.5	4	4.5	4	5.5	5
28	3	2	3	2	3	3	4
31	3	2	4.5	3.5	4.5	3	4
	2.73	2.55	3.68	3.50	3.68	3.82	4.00

PLANT	LRF5	RC41	RC41	RC41	516
	521	516	516	521	521
	535	521	535	535	535
10	3	2.5	3.5	2.5	3.5
24	4	1	2	2.5	2
8	3.5	4	3.5	4	5
23	3	4	2	2.5	3.5
2	2	1	2	2.5	2
4	1.5	2.5	2	2.5	0.5
18	4	2.5	3.5	2.5	3.5
5	0.5	2.5	2	1	2
26	0.5	2.5	2	2.5	0.5
39	3	2.5	3.5	2.5	3.5
20	0.5	2.5	2	1	2
	2.32	2.50	2.55	2.36	2.55
9	0.5	2.5	2	1	2
35	4	2.5	3.5	2.5	3.5
29	3	1	2	2.5	2
30	4	2.5	3.5	2.5	3.5
37	4.5	4	3.5	4	5
6	4	2.5	3.5	4	2
17	1.5	1	0.5	1	0.5
1	4	1	2	2.5	2
19	3	2.5	3.5	2.5	3.5
22	5.5	4	3.5	4	5
25	4	2.5	3.5	4	2
	3.45	2.36	2.82	2.77	2.82
34	3	4	5	4	3.5
33	4.5	5.5	5	5.5	5
3	1.5	2.5	2	1	2
11	5.5	4	3.5	4	5
32	3	2.5	3.5	2.5	3.5
13	5.5	4	3.5	4	5
14	3	2.5	3.5	4	2
16	4.5	4	3.5	4	5
21	5.5	4	3.5	4	5
28	3	2.5	3.5	2.5	3.5
31	3	4	5	4	3.5
	3.82	3.59	3.77	3.59	3.91